ultrasensitive asssays and as >0.1 ng/ml on conventional assays, respectively. However, not all patients who show PSA recurrence, based on ultrasensitive assays, develop clinical progression as well as an additional PSA recurrence over time (24,26). The determination of such a low level of PSA may be influenced by either the residual benign prostatic glands or non-prostatic tissues such as the periurethral glands (24–26). Therefore, there may be a risk in treating the residual benign glands only, if treatment is initiated at such a lower level of PSA. Furthermore, the patients' awareness of PSA rising even at extremely low levels has a considerable effect on the emotional quality of life, and there is no definite evidence that early intervention may decrease future morbidity and prolong the overall survival. The European Consensus Group (21) recommended that the ultrasensitive PSA assay should be used for monitoring patients but not for decision making (Table 1).

As a result, although there is no definite consensus regarding the PSA cut-off point for defining PSA recurrence after radical prostatectomy, a PSA level of 0.2 ng/ml on conventional assays seems to be the most acceptable cut-off point for PSA recurrence based on a clinical point of view.

FACTORS PREDICTING PSA RECURRENCE AFTER RADICAL PROSTATECTOMY

The local extent of disease on a DRE (T stage), serum PSA level and Gleason score from prostate biopsy specimens have all been considered to be important factors for predicting the pathological stage (pT stage) for patients with clinically localized prostate cancer (27,28). Regarding the pre-operative PSA level, Partin et al. (27) reported that 64, 50, 35 and 16% of patients with a serum PSA level <4, 4–10, 10–20 and >20 ng/ml, respectively, had pathologically organ-confined disease. Pelvic lymph node involvement is found in nearly 3, 9 and 17% of patients with a serum PSA level <10, 10–20 and >20 ng/ml, respectively. As a result, patients with a serum PSA level between 10 and 20 ng/ml are at an intermediate risk for PSA recurrence, while those with a serum PSA level >20 ng/ml represent a high-risk population for developing PSA recurrence after radical prostatectomy.

Regarding the Gleason score of biopsy specimens, Partin et al. (27) reported that 55, 29 and only 17% of the patients with a Gleason score of ≤6, of 7 and of ≥8 based on biopsy specimens, respectively, have pathologically organ-confined disease. Pelvic lymph node involvement is found in nearly 3, 10 and 20% of patients with a Gleason score of ≤6, of 7 and of ≥8, respectively. They (27,29,30) constructed a nomogram based on these pre-operative parameters (Partin tables) in the 1990s to assist urologists in pre-operatively predicting the final pathological stage. The Partin tables have recently been updated to reflect the dramatic change in the stage of prostate cancer at presentation during the past decade (28). Using the Partin tables, information regarding the probability of various pathological stages, such as organ-confined disease, extraprostatic extension, and seminal vesicle or lymph node involvement, is provided pre-operatively. Such pathological stages can serve as an excellent surrogate for the outcome after radical prostatectomy.

The Gleason score of radical prostatectomy specimens is also an important factor for predicting PSA recurrence after radical prostatectomy (29,31). The presence of a Gleason grade ≥4, or a Gleason score >7 on radical prostatectomy specimens is predictive of a high-risk for PSA recurrence (31–33).

Khan et al. (34) constructed a nomogram that was simple to use and divided the probability of long-term PSA recurrence-free survival into four groups according to the prostatectomy Gleason score, pathological stage and surgical margin status: namely, excellent, good, moderate and low (Table 2). Group 1 consists of patients who have an excellent PSA recurrence-free survival (95% at 10 years); they have a Gleason score of ≤6, organ-confined or extraprostatic extension of the disease and negative surgical margins. Group 2 includes patients who have a good PSA recurrence-free survival (72% at 10 years); they have a Gleason score of 7, organ-confined or extraprostatic extension of the disease and negative surgical margins, or a Gleason score of ≤6, organ-confined or extraprostatic extension of the disease and positive surgical margins. Group 3

Table 2. Estimation of 5 and 10-year likelihood of biochemical recurrence-free survival and four prognosis groups determined by pathological stage, surgical margin status and prostatectomy Gleason score (34)

Pathological stage	Gleason score	Surgical margin status	5-year bNED (%)	10-year bNED (%)	Prognosis group
OC or EPE	2–6	Negative	97 (95–98)	95 (92–96)	Excellent
OC or EPE	7	Negative	86 (82–90)	72 (62–80)	Good
OC or EPE	2–6	Positive			
OC	8–10	Positive/negative	62 (51–70)	41 (29–55)	Moderate
EPE	8–10	Negative			
EPE	7–10	Positive			
SV	2–10	Positive/negative			
LN	2–10	Positive/negative	37 (26–48)	13 (4–26)	Low

bNED, biochemical recurrence-free survival; OC, organ confined; EPE, extraprostatic extension; SV, positive seminal vesicles but negative lymph nodes; LN, positive lymph nodes. The numbers in parentheses are the 95% confidence intervals.

consists of patients who have moderate PSA recurrence-free survival (41% at 10 years); they have a Gleason score of 7–10 with extraprostatic extension and positive surgical margins, or a Gleason score of 8–10 with extraprostatic extension, or positive seminal vesicle involvement. Group 4 consists of patients who have a low PSA recurrence-free survival (13% at 10 years); they have disease involvement in the pelvic lymph nodes.

In addition to standard pathological examinations, various histopathological determinants and molecular markers have been evaluated to predict PSA recurrence and survival. Bauer et al. (35) reported the p53 tumor suppressor gene expression and bcl-2 protooncogene expression to be significant risk factors for PSA recurrence after radical prostatectomy. However, the predictive value of these molecular markers remains controversial (36,37). The expression of Ki-67 (36) and p27 (38), apoptotic index (36), DNA ploidy (39) and tumor angiogenesis (microvessel density) (40) have also been reported to be possible predictive factors of PSA recurrence after radical prostatectomy.

Table 3. Estimation of metastasis-free rates following PSA failure after radical prostatectomy (8)

Prognostic factors	Metastasis-free survival (%)		
	3 years	5 years	7 years
All men with PSA recurrence	78 (73–84)	63 (56–70)	52 (44–60)
Gleason score 5–7	86 (79–90)	73 (65–80)	62 (52–71)
PSA recurrence >2 years	89 (81–94)	82 (71–94)	77 (65–86)
PSA doubling time >10 months	95 (83–96)	86 (74–92)	82 (69–90)
PSA doubling time ≤10 months	82 (54–94)	69 (40–86)	60 (32–80)
PSA recurrence ≤2 years	80 (68–88)	62 (49–73)	47 (33–60)
PSA doubling time >10 months	79 (65–88)	76 (61–86)	59 (40–73)
PSA doubling time ≤10 months	81 (57–93)	35 (16–56)	15 (4–33)
Gleason score 8–10	63 (52–73)	40 (28–54)	29 (16–43)
PSA recurrence >2 years	77 (55–89)	60 (33–79)	47 (17–72)
PSA recurrence ≤2 years	53 (39–66)	31 (17–45)	21 (9–35)

PSA: prostate-specific antigen.
Numbers in parentheses are 95% confidence intervals.

NATURAL HISTORY OF PSA RECURRENCE

It has become apparent that the outcome of patients with PSA recurrence after radical prostatectomy is extremely heterogeneous, although there have only been a few reports providing direct information on the long-term natural history of PSA recurrence. Pound et al. (8) provided an excellent account of the natural history of PSA recurrence after radical prostatectomy by stratifying patients into varying risks for the development of metastatic disease or death. They (8) reviewed the outcome of 1997 patients, who received radical prostatectomy and pelvic lymphadenectomy by a single surgeon for clinically localized (stage T1, T2 and T3a) prostate cancer between 1982 and 1997, with a median follow-up of 5.3 years, and thus reported PSA recurrence to develop in 315 (15%) patients. In this series, the patients with PSA recurrence were observed until there was evidence of clinical metastatic progression, and then hormonal therapy was initiated. Eleven patients who received early hormonal therapy after an increase in their PSA level were excluded from the analysis. Of the remaining 304 patients, 103 (34%) developed metastatic disease. The median actuarial time from the development of PSA recurrence to the identification of metastases was 8 years, and the 5-year metastasis-free rate was 63%. The Gleason score on radical prostatectomy specimens (5–7 versus 8–10), the time from the radical prostatectomy to PSA recurrence (≤2 versus >2 years) and the PSA doubling time (PSADT; ≤10 versus >10 months) were all found to be predictive of the probability and time to the subsequent development of metastatic disease. To enhance its clinical applicability, they (8) developed an algorithm for estimating a patient's probability of remaining free of metastatic disease at 3, 5 and 7 years (Table 3). Using this algorithm, patients who are likely to have an indolent course can be identified and spared the potential morbidity of additional therapy. Conversely, patients with a high risk of disease progression can be identified early and thus more quickly be administered hormonal therapy (41,42). Although this algorithm undoubtedly provides the most comprehensive information to date, its availability will be enhanced even more by adding data concerning patients with a Gleason score from 8 to 10 and new parameters such as molecular markers.

Once patients developed metastatic disease, the median actuarial time to death was 5 years, and the cancer-specific survival at 10 and 15 years following surgery was 94 and 91%, respectively (8). The time interval from surgery to the development of metastatic disease was predictive of the time until death. Men who developed metastases within 1–3 years following surgery tended to die from cancer at a higher rate than those who developed metastases >4 years after surgery.

SITE OF RECURRENCE

It is important to distinguish whether an increase in the PSA level after radical prostatectomy is due to local recurrence, distant metastases or a combination of both, because the management regimen is determined according to the recurrence pattern. Pound et al. (4) reported that approximately one-third of the patients who eventually developed clinical recurrence had local evidence of disease and 70% had distant metastasis with or without local recurrence. Other investigators also estimated a low probability for local recurrence, ranging between 10 and 25% (11,43).

Many approaches have been attempted to identify the site of recurrence. Regarding a DRE, several investigators have demonstrated that >50% of the patients with biopsy-proven local recurrence have no abnormalities on DRE (44–46). Lightner et al. (47) mentioned that an induration in the prostatic fossa may be secondary to a benign scar rather than

malignancy. As a result, DRE is considered not to be very helpful in determining the site of recurrence (48). Despite its low sensitivity, however, serial DREs are non-invasive and cheap and thus may be potentially helpful in detecting subtle changes that may reflect local recurrence.

The usefulness of transrectal ultrasound (TRUS)-guided anastomotic biopsies is also unclear. Several studies have demonstrated the sensitivity of this technique to be quite poor in patients with a PSA <1.0 ng/ml, at which level salvage radiotherapy is most efficatious (46,49,50). Shekarriz et al. (50) found only 25% of the patients with a PSA ≤1.0 ng/ml to have a positive biopsy compared with 71% of those with a PSA >1.0 ng/ml. Furthermore, a positive anastomotic biopsy is not associated with an improved outcome after salvage radiotherapy (51) and 10–40% of the patients with a negative biopsy and a PSA <1.0 ng/ml show a PSA decrease after salvage radiotherapy, thus suggesting the presence of undetected local recurrence (52). As a result, since a negative biopsy does not always rule out local recurrence, and a positive result does not always exclude the presence of metastatic disease, the role of anastomotic biopsies remains ambiguous.

There is no imaging test to identify recurrent lesions accurately in patients demonstrating lower PSA levels. Cher et al. (53) found the probability of a positive bone scintigram to be <5% until the PSA value increased to 40–45 ng/ml. They concluded that serum PSA is the best predictor of the bone scintigram results in patients with rising serum PSA levels after radical prostatectomy, and bone scintigraphy is only of limited usefulness until the PSA level increases to >30–40 ng/ml. There is no consensus concerning the PSA level at which a bone scan should be performed, but recently a delay was recommended until the serum PSA reached 20 ng/ml, provided that the patient was asymptomatic. Despite the small likelihood of a positive finding, however, an evaluation by early bone scan may be necessary as a baseline for comparison purposes with future studies that are performed as the serum PSA ultimately continues to increase.

Computed tomography (CT) scans are not sufficiently sensitive for detecting local recurrence until the increasing rate of PSA becomes >20 ng/ml per year (54). The sensitivity and specificity of magnetic resonance imaging (MRI) and MR spectroscopy are improving and they are most useful for detecting nodal and bony metastases (55,56). However, they are also not sufficiently useful early in the course of PSA recurrence. Positron emission tomography (PET), a biochemical imaging modality, cannot accurately distinguish post-operative scars from local recurrence (42). Immunoscintigraphy, a technique in which a radiolabelled monoclonal antibody against prostate-specific membrane antigen (PSMA) is used to bind to PSMA, is now being increasingly used to evaluate patients with a rising serum PSA after radical prostatectomy. By combining the results of Levesque et al. (57) and Kahn et al. (58), Lange et al. (59) showed promising data in which the response to salvage radiotherapy was 28% when scans revealed extraprostatic disease; however, this value rose to 70% when scan results demonstrated either activity in the prostatic fossa only or a normal scan. In those studies, however, the PSA level was high at the time of scanning. As a result, the true usefulness of this test in patients demonstrating a lower PSA level, when radiotherapy has the most potential to be beneficial, is unclear. This new technique is still in its early phase of use and further studies are required to evaluate its usefulness.

In view of the limited role of such imaging tests to identify the site of recurrence, statistical models based on various clinical and pathological risk factors have been developed. Cadeddu et al. (60) reported that of 82 patients treated with radiation therapy for PSA recurrence, the patients with Gleason score ≥8, positive seminal vesicles or lymph nodes, or a PSA recurrence within the first year following surgery rarely benefit from radiation therapy. This finding suggests that PSA recurrence in such patients may be due to distant metastases or a combination of distant metastases and local recurrence. Conversely, PSA recurrence is more likely to be due to local recurrence alone if there is a Gleason score ≤7 or an absence of nodal or seminal vesicle involvement. Furthermore, Kupelian et al. (2) reported that surgical margin involvement was the only independent predictor of local failure. Partin et al. (61) mentioned that a serum PSA velocity ≥0.75 ng/ml/year was associated with an increased likelihood of metastatic disease. They concluded that the combination of the Gleason score, pathological stage and serum PSA velocity 1 year after surgery best distinguished local recurrence from distant metastases. Patel et al. (62) demonstrated that a PSADT of <6 months was most indicative of distant metastases, whereas local recurrence correlated with a long PSA doubling time. Trapasso et al. (6) reported the median PSADT to be 4.3 months for patients who were ultimately found to have metastatic disease compared with 11.7 months for patients with local recurrence alone. Pound et al. (8) demonstrated that PSADT (≤10 months), Gleason score (>7) and time to PSA recurrence (≤2 years) were important in determining the probability of progression to distant metastases thereafter. Many studies therefore suggest that patients who develop PSA recurrence within 1–2 years of surgery, have a Gleason score of >7, positive seminal vesicles or lymph node involvement are more likely to have metastatic disease and are thus considered to be better candidates for systemic treatment (Table 4) (2,4,8,49,60,63,64). For further confirmation, however, prospective studies concerning PSA parameters are necessary.

TREATMENT OF PSA RECURRENCE

The best way to treat PSA recurrence after radical prostatectomy may depend on the site of recurrence: namely local, systemic or a combination of both. The treatment options for presumed local recurrence include external beam radiotherapy and, for presumed distant metastasis, hormonal therapy. Observation only is also one of the treatment options regardless of the recurrence site. However, standard imaging

Table 4. Summary of clinicopathological factors that predict local or distant recurrence

	Local recurrence	Distant recurrence	Reference
PSADT	>6 months	≤6 months	62
		<10 months	8
PSA velocity	<0.75 ng/ml/year	≥0.75 ng/ml/year	61
Time from RP to PSA recurrence	≥1 year	<1 year	60, 63
		≤2 years	4, 8
Gleason score on RP specimens		8–10	4, 8, 60
Surgical margin involvement	(+)		2
SV or LN involvement	(−)	(+)	4, 49, 60, 64

PSA, prostate-specific antigen; PSADT, doubling time; RP, radical prostatectomy; SV, seminal vesicle; LN, lynph node.

tests cannot help to identify the site of recurrence until the PSA reaches 20–50 ng/ml, at which level the effectiveness of radiotherapy can no longer be expected. Therefore, treatment is mainly selected according to the pathological findings of the radical prostatectomy specimen and the post-operative serum PSA parameters.

Observation

According to a report by Pound et al. (8), the natural course from PSA recurrence to the development of metastatic disease or prostate cancer-specific death seems to be quite long. Frazier et al. (65) mentioned that the majority of patients (93%) with PSA recurrence had not failed clinically and concluded that PSA recurrence may not translate into disease-related death. As a result, observation with delayed hormonal therapy for symptomatic or metastatic disease can be one of the treatment options. According to the international survey on the management of PSA recurrence after radical prostatectomy, 54% of urologists preferred observation, whereas 31% opted for hormonal therapy and only 13% selected salvage radiotherapy (66).

Radiation Therapy

Salvage radiotherapy is the recommended terminology for curative-intended radiation for post-operative PSA recurrence as opposed to adjuvant radiotherapy administered shortly after radical prostatectomy based on adverse pathological findings (67). To be candidates for salvage radiation therapy, patients must have a life expectancy of >10 years, since the salvage radiation therapy is sometimes associated with high morbidity.

The PSA response to radiotherapy for PSA recurrence varies from 18 to 68% (68–71,73). The PSA level before radiation is critical in the response to salvage radiotherapy (69, 71–74). Schild et al. (71) reported patients with PSA levels of ≤1.1 ng/ml at the beginning of radiotherapy to have a 30 month actuarial freedom from failure of 78% in comparison with only 18% for those with higher pre-treatment PSA levels. Kooy et al. (72) reported the 8-year relapse-free survival of patients who received salvage radiotherapy to be 67, 39 and 42% in patients with a pre-radiotherapy PSA level of ≤1.0, 1.1–4 and >4 ng/ml, respectively. Nudel et al. (73) reported that patients who received salvage radiotherapy at PSA <1 ng/ml after radical prostatectomy and those who received radiotherapy as an adjuvant treatment to surgery had equivalent progression-free survival, but it was significantly worse if radiotherapy was delayed until the PSA reached a level >1 ng/ml. These reports suggest that a PSA cut-off point of 1 ng/ml is likely to confer the best chance of biochemical survival. Garg et al. (69) reported the 3-year disease-free survival rate to be 78% in patients with a PSA level of ≤2 ng/ml at the time of radiotherapy compared with 31% in those with a PSA level >2 ng/ml. Peschel et al. (74) reported the pre-operative PSA level, pre-radiotherapy PSA level and seminal vesicle involvement to be significant risk factors for actuarial biochemical disease-free survival following post-operative radiotherapy, and the most significant risk factor was the pre-radiotherapy PSA of >0.3 ng/ml. The American Society for Therapeutic Radiology and Oncology (ASTRO) Consensus Panel demonstrated a serum PSA level of 1.5 ng/ml to be the threshold level for optimal success rates (67). As most recently recommended by the European Consensus Group (21), a PSA level of 1.0–1.5 ng/ml is considered to be an appropriate cut-off point to initiate salvage radiotherapy for presumed local recurrence.

The dose of radiation is also an important factor influencing the response to PSA recurrence after radical prostatectomy. Schild et al. (71) reported that patients who received ≥64 Gy had a 30 month freedom from failure of 62% in comparison with 17% for those who had a smaller dose. The ASTRO Consensus Panel recommended that at least a dose of 64.8 Gy radiation should be administered to the prostatic bed (67). The European Consensus Group (21) also recommended that the minimum dose that should be delivered is 64 Gy with 1.8 or 2 Gy per fraction.

The response to salvage radiotherapy for PSA recurrence after radical prostatectomy may depend on the site of recurrence. Katz et al. (75) reported negative/close margins, an absence of extracapsular extension and the presence of seminal vesicle invasion to be independent predictors of PSA relapse following salvage conformal radiotherapy for PSA recurrence. Stephenson et al. (76) also reported a Gleason score of 8–10, a pre-radiotherapy PSA level >2.0 ng/ml, negative surgical margins, a PSA doubling time of ≤10 months and seminal vesicle invasion to be a predictor of disease progression following salvage radiotherapy. Therefore, patients with such clinicopathological characteristics may not be good candidates for salvage radiotherapy. Conversely, the long-term response may be expected for patients without such characteristics. However, further prospective studies are required to identify the candidates who can most benefit from salvage radiotherapy.

Hormonal therapy may increase the sensitivity to irradiation. Bolla et al. (77) showed that adjuvant hormonal therapy improved local recurrence, PSA-free survival and overall survival. Eulau et al. (78) also demonstrated that transient androgen deprivation around the time of salvage radiation therapy showed an improvement in the biochemical and clinical response rates. Katz et al. (75) also reported that neoadjuvant androgen deprivation improved the PSA relapse-free survival after salvage conformal radiotherapy in patients with any of the following factors, namely positive margins, extracapsular extension or seminal vesicle invasion. Androgen deprivation may be effective for possible distant metastases in such patients. However, the European Consensus Group (21) mentioned that hormonal therapy is not standard in patients receiving salvage radiotherapy (Table 1). A prospective randomized study is necessary for an accurate evaluation of the role of androgen derivation combined with salvage radiation therapy.

When counseling patients regarding the use of salvage radiation therapy after a radical prostatectomy, it is important to keep in mind potential complications, such as gastrointestinal symptoms, new or worsened urinary incontinence and erectile dysfunction, associated with this therapy, although the incidence of severe long-term toxicity is uncommon. Tsien et al. (79) reported that using three-dimensional conformal radiotherapy at a median dose of 64.8 Gy, the 5 year actuarial likelihood of grade ≥2 rectal toxicity was 8.9%. Peyromaure et al. (80) also reported that irritative urinary disorders, hematuria and rectal irritation were observed in 9.7, 8.1 and 6.4% of patients who received salvage radiotherapy at a dose of 65 Gy, but none of them was severe. However, since these findings are based on the findings of a retrospective study, the incidence reported may be an underestimation of the actual complication rate (81). Prospective quality of life studies are necessary to make a more precise evaluation.

In conclusion, the role of salvage radiotherapy in the management of PSA recurrence after radical prostatectomy remains inconclusive.

HORMONAL THERAPY

Although androgen deprivation therapy by surgical (82) or medical castration using a luteinizing hormone-releasing hormone (LH-RH) agonist (83,84) or antiandrogens (85–87) has been widely used for the treatment of prostate cancer, the early use of such hormonal therapy for PSA recurrence after radical prostatectomy remains controversial. It has been extensively debated regarding whether or not giving early hormonal treatment is of any benefit compared with delayed treatment applied only when symptomatic progression occurs. The PSA level at which hormonal therapy should be initiated remains to be elucidated. Messing et al. (88) compared immediate versus deferred androgen deprivation therapy with surgical or medical castration by LH-RH agonist in patients who underwent radical prostatectomy and pelvic lymphadenectomy and were found to have nodal metastases. They demonstrated that immediate hormonal treatment led to a better overall survival, prostate cancer-specific survival and also progression-free survival. The aim of this study focused on the significance of adjuvant hormonal therapy for patients at high risk of disease progression after radical prostatectomy, but not on the significance of treatment for those with PSA recurrence after radical prostatectomy. However, this result suggests the possible survival benefit by androgen deprivation therapy for the treatment of PSA recurrence after radical prostatectomy.

Recently, Wirth et al. (89) reported the results of an interim analysis of the Early Prostate Cancer (EPC) program which consists of three randomized, double blind, placebo-controlled trials prospectively designed for combined analysis. In this program, a total of 8113 patients with localized or locally advanced prostate cancer were randomized to a pure antiandrogen (bicaltamide 150 mg/day) group or a placebo group in addition to standard care including watchful waiting, radical prostatectomy and radiation therapy. At a median 5.4 years of follow-up, a significant benefit due to bicaltamide in the progression-free survival was demonstrated in radical prostatectomy patients with locally advanced disease. Bicaltamide provides a similar survival outcome to castration including a bilateral orchiectomy or LH-RH agonist in previously untreated patients with locally advanced prostate cancer, and confers a statistically significant benefit over castration with respect to sexual interest and physical capacity (85,86). Another recent study comparing flutamide, another non-steroidal antiandrogen, versus no adjuvant treatment also showed that flutamide induced a better recurrence-free survival after radical prostatectomy for locally advanced, lymph node-negative prostate cancer with a median follow-up of 6.1 years, although there were no differences in terms of overall survival, and considerable toxicity was also observed in the flutamide arm (90). Since local recurrent lesions at an early stage of PSA recurrence is considered to be quite small, pure antiandrogens may be sufficient to prevent disease progression. Recently, the Japan Clinical Oncology Group (91) started a randomized controlled trial (JCOG 0401) to evaluate radiotherapy ± hormonal therapy with bicaltamide versus hormonal therapy alone for PSA recurrence after radical prostatectomy. The usefulness of bicaltamide or irradiation for the treatment of PSA recurrence after radical prostatectomy is thus expected to be clarified.

In order to avoid the side effects of hormonal therapy, the concept of the intermittent administration of hormonal therapy has been advocated (92). Despite the potential benefits of intermittent hormonal therapy, its long-term efficacy remains to be demonstrated. Confirmation of the efficacy of intermittent hormonal therapy by controlled clinical trials in comparison with standard consecutive hormonal therapy may be necessary before we can clinically recommend this treatment for PSA recurrence after radical prostatectomy. From the view point of side effects, the 5α-reductase inhibitor, finasteride, has recently attracted much attention. Finasteride may have an ability to delay disease progression by itself in patients with PSA recurrence after radical prostatectomy (93). However,

further confirmation by longer and larger studies is required for the use of finasteride as a treatment option for patients with PSA recurrence after radical prostatectomy.

Eventually, the treatment of PSA recurrence by suspected distant metatstasis involves hormonal manipulation, but at present the type and timing of such treatment are still based on physician and patient preference.

CONCLUSIONS

A significant proportion of patients who undergo a radical prostatectomy for localized prostate cancer develop PSA recurrence. In terms of the treatment for such PSA recurrence, some patients may be good candidates for local radiotherapy, whereas others may be indicated to undergo hormonal manipulation rather than radiotherapy. Although the pathological findings and post-operative serum PSA parameters may be useful for predicting the pattern of recurrence, it is still quite difficult to identify the most appropriate candidates for each type of treatment. The optimal type and timing of hormonal manipulation have yet to be elucidated. Further prospective randomized trials are thus still necessary to reach a consensus regarding the ideal treatment protocols for PSA recurrence after radical prostatectomy.

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Murine Androgen-Independent Neuroendocrine Carcinoma Promotes Metastasis of Human Prostate Cancer Cell Line LNCaP

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BACKGROUND. Although neuroendocrine (NE) cells in prostate cancer have been speculated to accelerate the growth and progression of surrounding cancer cells, the evidence is as yet inconclusive. We investigated the effect of an NE allograft (NE-10) and its cell line, NE-CS, which were established from the prostate of the LPB-Tag 12T-10 transgenic mouse, on human prostate cancer cell line LNCaP.
METHODS. The proliferation and pulmonary metastasis of LNCaP xenografts in athymic mice with and without NE-10 allografts were evaluated. Boyden chamber assay and microarray analysis were performed to investigate changes in invasion/migration and mRNA of LNCaP cells under the influence of the NE cells, respectively.
RESULTS. NE-10 did not influence the proliferation of LNCaP. The pulmonary metastasis of LNCaP with NE-10 significantly increased compared to mice without it. The NE-CS cells accelerated the in vitro invasion/migration of adenocarcinoma cells. Increased expression of mRNA of gelsolin was observed in LNCaP cells incubated with the supernatant of NE-CS cells.
CONCLUSIONS. The NE-10 allograft promotes pulmonary metastasis of subcutaneously inoculated LNCaP cells by facilitating cell invasion. Secretions from NE cells upregulate the expression of gelsolin, which is an actin-binding protein, resulting in acceleration of the migration of LNCaP cells. *Prostate 66: 536–545, 2006.* © 2005 Wiley-Liss, Inc.

KEY WORDS: **prostate cancer; neuroendocrine; gelsolin**

INTRODUCTION

To elucidate the molecular factors that lead to loss of androgen-dependence as well as the progression of prostate cancer has become one of the major tasks of current research on prostate cancer. Recently, it has focused on epigenetic events and cellular interactions between cancer cells and the surrounding cells. The role of neuroendocrine (NE) differentiated cells in prostate cancer in particular has attracted a great deal of attention. NE cells are identified as a component of conventional prostatic adenocarcinoma, occurring in 30–100% of tumors [1,2]. Androgen ablation induces an increased number of NE cells in prostate cancer [3] and the frequency and density of NE cells are more pronounced in hormone-refractory prostate cancer [4]. Since NE cells frequently lack androgen receptors (AR), they do not respond to androgen ablation therapy. However, NE cells do secrete growth-modulating

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neuropeptides. These observations have led to the hypothesis that NE cells are providers of paracrine factors that accelerate the growth and progression of surrounding prostate cancer cells toward an androgen-independent state. However, the relationship between NE cells and prostatic adenocarcinoma cells remains obscure. It is controversial whether the appearance of NE cells indicates a poor prognosis due to their ability to promote the progression of adenocarcinoma to androgen independence and metastasis [4,5].

We recently developed a transgenic mouse model of prostate cancer composed of NE carcinoma, using a recombinant gene expressing an SV40 large T-antigen (Tag) transforming sequence under the regulatory control of the rat large probasin promoter (LPB) [6]. An NE-10 allograft that is transplantable into athymic mice was established from a ventral lobe of the murine prostate [7]. This allograft exhibits NE features histologically and immunohistologically. Cells in the NE-10 allograft are weakly positive or negative for AR and the tumor shows androgen-independent growth in vivo. In addition, the in vitro cell line NE-CS was established from the NE-10 allograft [8]. The cells have characteristics of NE differentiation similar to the NE-10 allograft except they no longer express Tag. Transplantation of NE-CS cells into athymic mice indicates their tumorigenic ability.

We have reported that the NE-10 allograft promotes LNCaP (human prostate adenocarcinoma cell line) tumor progression to androgen independence [9]. LNCaP tumor growth decreases in mice bearing LNCaP alone after castration. In contrast, LNCaP tumors continue to grow in those bearing both LNCaP and NE-10 tumors. On the other hand, it remains unknown whether NE-10 is involved in the progression of prostatic adenocarcinoma in normal androgen status. In this study, we investigated whether the NE-10 allograft and NE-CS cell line affected the proliferation, adhesion, migration, invasion, and metastatic potential of LNCaP.

MATERIALS AND METHODS

Cell Cultures

The human prostate adenocarcinoma cell line LNCaP was obtained from the American Type Culture Collection, and used with passage numbers between 44 and 49. The murine prostate neuroendocrine cancer allograft (NE-10) and its cell line (NE-CS) were established from the LPB-Tag transgenic mouse line 12T-10 [7,8]. The LNCaP and NE-CS cells were maintained in culture medium [RPMI-1640 (Gibco BRL, Breda, The Netherlands) supplemented with MEM non-essential amino acid (10 ml/L, Gibco BRL), MEM sodium pyruvate, penicillin-streptomycin (10 ml/L, Gibco BRL), and containing 10% fetal bovine serum (FBS, ICN Biomedicals, Costa Mesa, CA), and 7.5% $NaHCO_3$] in 5% CO_2 in a humidified incubator.

In Vivo Study

LNCaP cells (5×10^6 suspended in 75 µl of serum-free RPMI-1640 medium) were mixed with 75 µl of Matrigel (Becton Dickinson, Sunnyvale, CA) in a syringe. These cells were subcutaneously injected into the backs of 6-week-old athymic male mice (Balb/c, nu/nu, Sankyo Labo, Tokyo, Japan). At 7 weeks after injection, the mice with LNCaP xenografts were randomly divided into two groups (LNCaP-only and LNCaP+NE-10). In the LNCaP+NE-10 group, a 50 mg tissue block of the NE-10 allograft was inoculated into the contralateral back of each mouse with LNCaP. The tumor volume of the LNCaP xenograft was measured every other week. The tumor volume (mm^3) of LNCaP was calculated by the formula $0.523 \times$ long diameter $(mm)^2 \times$ short diameter (mm). At 17 weeks after injection of LNCaP cells, the mice were killed. Blood samples were taken from the ventricle, and the lungs were removed. Bromodeoxyuridine (BrdU, Roche Molecular Biochemicals, Mannheim, Germany), 1 ml/ 100 g (body weight), was administered into the abdominal cavity of each mouse at 1 hr before sacrifice.

The lung tissues were fixed in 10% formalin and embedded in paraffin to assess incorporation of BrdU and immunohistochemistry for prostate-specific antigen (PSA) and Tag. Microscopic images of the PSA immunostained sections were obtained using a digital camera equipped with a microscope. The images of each specimen with the largest area were processed as the whole area of the lung for analysis using NIH Image (National Institutes of Health, Bethesda, Maryland).

Serum levels of PSA were measured in blood from the mice with the NE-10 allograft and control mice by using the radioimmunoassay Tandem-R PSA (SRL, Tokyo, Japan).

Immunohistochemistry for PSA, T-Antigen, BrdU, and CD31.

Tissue sections were stained with an antibody against Tag using a technique reported previously [6]. PSA and CD31 immunostaining was performed using a rabbit polyclonal anti-human antibody against PSA (Code No. A0562, 1:2000, DakoCytomation, Glostrup, Denmark) and monoclonal anti-human antibody against CD31 (Code No. M0823, 1:40, DakoCytomation), respectively. BrdU immunostaining was performed using a 5-Bromo-2'-deoxy-uridine Labeling and Detection Kit II (Roche, Mannheim, Germany) according to the manufacturer's instructions.

Preparation of the Medium for In Vitro Studies

The supernatant of NE-CS cells that were incubated in culture medium in 5% CO_2 in a humidified incubator for 36 hr was used as NE medium. Culture medium without NE-CS cells that was left in culture dish under the same conditions for 36 hr was prepared as control medium. NE medium and control medium were used for cell proliferation assay, cell migration/invasion assay, cell adhesion assay, microarray analysis, Northern blot analysis, and florescence staining for actin filaments.

Cell Proliferation Assay

LNCaP cells (2×10^4 cells/well) were suspended in wells of a 96-well plate. At 3 days after suspension in culture medium, it was changed to NE medium (n = 9) or control medium (n = 9). After culture for an additional 3 days, MTT assay was performed for estimating cellular viability using a commercially available kit (Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan).

Cell Migration and Invasion Assay

In vitro invasiveness of LNCaP was determined according to the method described by Albini et al. [10]. Briefly, in a Transwell culture chamber (Coster Science, Cambridge, MA), a polyvinylpyrrolidone-free polycarbonate filter with an 8.0 μm pore size was precoated with 5 μg of fibronectin (Biomedical Technologies, Stoughton, MA) on the lower surface and 10 μg of the reconstituted basement membrane material Matrigel (Becton Dickinson, Sunnyvale, CA) on the upper surface. The cells that invaded across the pores were counted under a microscope after hematoxylin and eosin staining. The experiments were carried out in triplicate. Cell migration assay was performed using the filter without Matrigel coating.

Two different experiments were performed. In Experiment 1, LNCaP cells (1×10^5) were harvested and placed in the upper chamber of a Boyden chamber with 100 μl of NE medium or control medium. In the lower chamber, 600 μl of culture medium was added. In Experiment 2, 600 μl of culture medium with or without NE-CS cells was incubated in the lower chamber for 36 hr and then LNCaP cells (1×10^5) suspended in 100 μl of culture medium were placed in the upper chamber. The numbers of migrating and invading cells were counted at 2, 4, 6, and 8 hr and 4, 8, 12, and 20 hr, respectively.

Cell Adhesion Assay

Harvested LNCaP cells (1×10^3) were resuspended in NE medium or control medium and plated in 24-well plates precoated with collagen type IV (Becton Dickinson, Franklin Lakes, NJ) and incubated for 1 or 2 hr. Non-adherent cells were removed by aspiration, and washed three times with PBS. The total protein content of adherent cells was determined by the BCA method (Pierce, Rockford, IL). The absorbance was determined at a wavelength of 562 nm in a model microplate reader in triplicate experiments.

Isolation of RNA and Microarray

LNCaP cells cultured in culture medium were harvested when they reached subconfluence. The LNCaP cells suspended in the NE medium or control medium were incubated. Before (0 hr) and after treatment with NE medium or control medium for 4 hr and 8 hr, total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA microarray analysis was conducted by Kurabo Corp. Target preparation, CodeLink™ DNA microarray hybridization (Amersham Bioscience, Piscataway, NJ), and processing were performed as described previously [11]. The fragmented target cRNA was used for hybridization of each UniSet Human 1 Expression Bioarray chip. In this analysis, 1.5-fold up or downregulation was defined as a significant change.

Northern Blot Analysis

To confirm the reliability of results obtained from the microarray, Northern blot analysis was performed using total RNA derived from LNCaP cells at 4 hr and 8 hr after incubation with NE medium or control medium. Electrophoresis, blotting, and hybridization were done according to a standard protocol. Complementary DNA probes were generated by RT-PCR from total RNA of LNCaP cells. After amplification by the conventional PCR technique (*Gelsolin*: forward 5′-ACTGGTCTACTGTGTCTCTA-3′ and reverse 5′-TCTTCAGCCCACACTTTCTG-3′), the PCR products were purified using a Gel Extraction Kit (Qiagen) and a random hexamer labeled with alkaline phosphatase (Amersham AlkphosDirect). The hybridization signals were detected with CDP-Star Chemiluminescent Detection Reagent and exposed to hyperfilm (Amersham). Ethidium bromide staining of 28 S RNA was used to compare the amounts of loading.

Fluorescence Staining for Actin Filaments

Harvested LNCaP cells were resuspended in NE medium or control medium and incubated in a 2-well culture slide precoated with fibronectin for 5 hr (Becton Dickinson, Franklin Lakes, NJ). After discarding the medium, adherent cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 for

5 min, and stained with 0.2 μg/ml FITC-labeled phalloidin (Sigma, St. Louis, MO), which binds actin filaments for 20 min. After washing, the slides were coversliped and observed under a Zeiss LSM510 confocal microscope (Carl Zeiss, Inc., Thornwood, NY).

Statistical Analysis

Statistical analysis was performed with StatView 4.5 (Abacus Concepts, Inc., Berkeley, CA). The Mann–Whitney *U*-test was applied to compare results between two different groups. Repeated-measures ANOVA was used when comparing the in vitro cell migration and invasiveness in an individual group. Statistical significance was assigned at $P < 0.05$.

RESULTS

NE Cells Have no Effect on the Proliferation of Adenocarcinoma Cells

To investigate whether the NE-10 allograft altered the growth of the LNCaP xenograft in vivo, the allograft was co-transplanted into the contralateral back in athymic mice with the LNCaP xenograft. There was no significant difference in the tumor size of LNCaP xenografts between the mice with NE-10 and those without NE-10 for 10 weeks (Fig. 1A). The supernatant of NE-CS cells (NE medium) did not affect the growth of LNCaP cells in vitro on MTT assay (Fig. 1B), which was consistent with the result of the in vivo study. The levels of serum PSA were 189 ± 86 ng/ml in the mice with NE-10 and 260 ± 287 ng/ml in the mice without NE-10 (mean ± standard deviation), with no significant difference between the two groups. These results indicated that NE cells had no effect on the growth of LNCaP cells in vitro or in vivo.

NE Cells Promote Metastasis of Adenocarcinoma Cells

We examined whether the NE-10 allograft influenced the metastatic ability of LNCaP cells. PSA-positive cells, which indicated LNCaP cells in the lungs, were detected in six of nine mice in the LNCaP-only group, and in five of nine mice in the LNCaP + NE-10 group (Fig. 2A). Although, it has been reported that metastasis from the subcutaneously inoculated LNCaP xenograft is extremely rare if examined by routine histological examination with hematoxylin and eosin staining [12], careful examination by PSA immunostaining detected PSA-positive cells in lungs in the LNCaP-only group. Another set of experiments using a different lot number of LNCaP cells showed similar results (data not shown). The number of the clusters of the PSA-positive cells in the LNCaP + NE-10 group

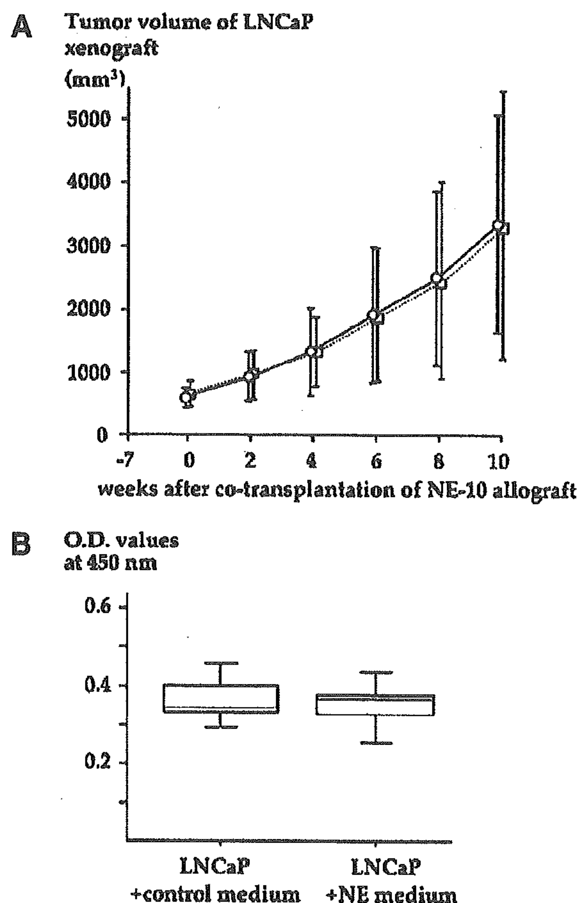

Fig. 1. **A**: Growth rate of LNCaP xenograft in athymic male mice. LNCaP cells (5×10^6) with Matrigel were subcutaneously injected into the backs of 6-week-old athymic mice. At 7 weeks after injection, the mice were randomly divided into two groups (open box, LNCaP-only, n = 9; open circle, LNCaP + NE-10, n = 9). For the LNCaP + NE-10 group, a 50 mg tissue block of the NE-10 allograft was inoculated into the contralateral back in mice with the LNCaP xenograft. Tumor volume (mm^3) is represented as the mean of nine mice in each group. Vertical bars indicate standard deviations. There was no significant difference in the size of the LNCaP xenograft between the two groups. **B**: Proliferation of LNCaP cells in an in vitro study. Cell proliferation was evaluated using MTT assay. There was no significant difference in optical density between LNCaP cells in NE medium and those in control medium ($P = 0.531$, Mann–Whitney *U*-test). Box presents 25th–75th percentiles, as well as median (center line). Bars indicate 5th and 95th percentiles. O.D., optical density.

was significantly greater than that in the LNCaP-only group ($P = 0.012$, Fig. 2B-1). Likewise, the total area of PSA-positive cells per whole lung was significantly larger than that in the LNCaP-only group ($P = 0.012$, Fig. 2B-2). The PSA-positive cells in the LNCaP + NE-10 group were not located adjacent to the Tag-positive cells representing metastases from the NE-10 allograft (Fig. 3).

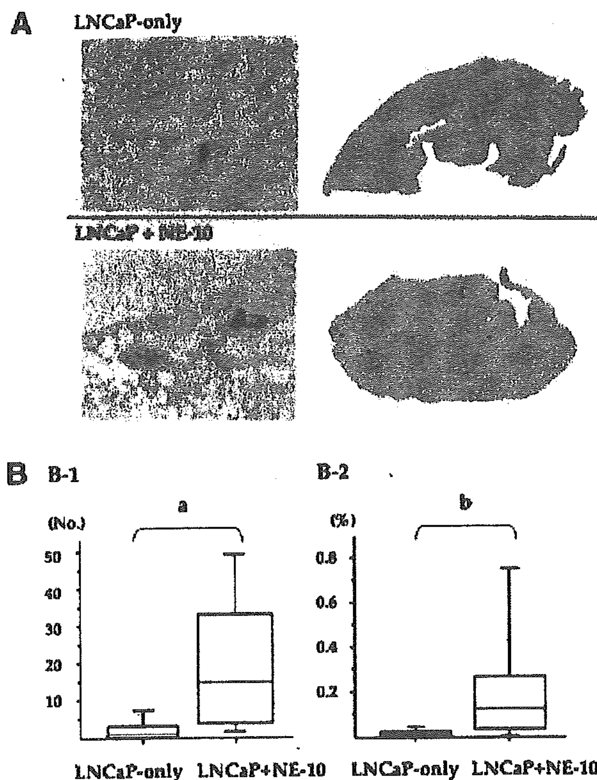

Fig. 2. **A**: Representative NIH images and immunohistochemistry for PSA. The metastatic ability of LNCaP affected by the NE-10 allograft was evaluated in an in vivo study. The LNCaP cells in the lungs of the athymic mice were detected using immunohistochemistry for PSA (**left panels**). Images of PSA-immunostained slides were obtained by digital camera and converted to NIH images (**right panels**). Black areas in NIH images show clusters of PSA-positive cells per unit lung area. Many clusters of PSA-positive cells were observed in the LNCaP + NE-10 group compared to the LNCaP-only group. The clusters of PSA-positive cells in the LNCaP + NE group were generally larger than those of the LNCaP-only group. Reduced from ×200. **B**: Quantitative analysis of the number of clusters of PSA-positive cells (B-1) and the percentage of PSA-positive cells per whole lung section (B-2). The number and percentage of clusters of PSA-positive cells per whole lung section were quantified. The PSA-positive clusters in the LNCaP + NE-10 group were significantly greater in number (B-1) and extent (B-2) than those of the LNCaP-only group (a, $P = 0.012$; b, $P = 0.012$; Mann–Whitney *U*-test). Box presents 25th–75th percentiles, as well as median (center line). Bars indicate 5th and 95th percentiles.

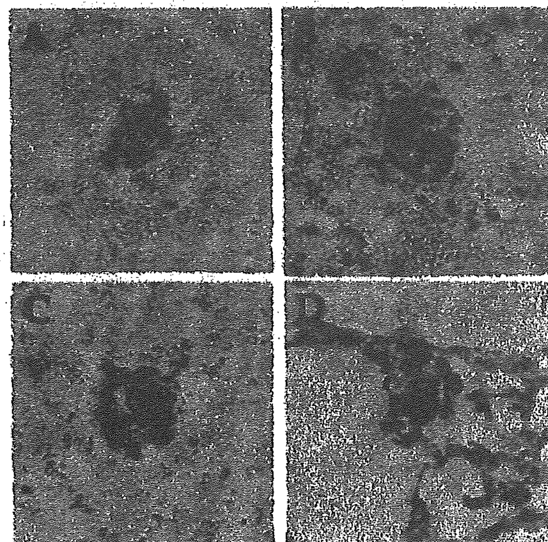

Fig. 3. **A**: Immunohistochemistry for PSA; (**B**) Immunohistochemistry for T-antigen; (**C**) Immunohistochemistry for BrdU; (**D**) Immunohistochemistry for CD31. (A, B, and C were in serial sections). The clusters of PSA-positive cells show positive staining for BrdU. The PSA-positive cells (LNCaP) do not neighbor Tag-positive cells (NE cells). Some clusters of PSA-positive cells lacked CD31-positive endothelial cells (arrow). The results indicated that the PSA-positive cells, which had proliferative activity accompanied by escape from the vascular lumina into the alveoli, were metastases from the LNCaP xenografts that were inoculated into the backs of athymic mice. Reduced from ×400.

We examined whether the PSA-positive cells in the lungs were metastases or just tumor emboli of the cells from the LNCaP xenograft. The nuclei of the PSA-positive cells in serial sections were stained a uniform, dense blue color on BrdU immunohistochemistry without Tag expression (Fig. 3A–C). Immunostaining for CD31 showed that some clusters of PSA-positive cells lacked the surrounding CD31-positive endothelial cells (Fig. 3D). On the other hand, obvious invasion into the alveoli was not detected when the clusters of PSA-positive cells were relatively small. Thus, not all clusters of PSA-positive cells in the lungs were metastases defined as lesions accompanied by escape from the vascular lumina into the alveoli in the present study. However, if we maintained the mice for more than 17 weeks after the inoculation of LNCaP cells, the small tumor emboli that are prerequisite for metastatic development might have become apparent metastases as their tumor size increased since the PSA-positive cells in the lungs had proliferative ability and LNCaP cells possessed invasive potential and could enter into the vascular circulation at the subcutaneously inoculated primary site. Furthermore, it was unlikely that mechanical destruction of the pulmonary tissues by NE-10 metastasis provided a scaffold for LNCaP metastasis because no Tag-positive cells (NE-10 cells) were observed around the metastatic nodules of LNCaP. Thus, the NE-10 allograft promoted pulmonary metastasis of the subcutaneously inoculated LNCaP xenograft.

NE Cells Accelerate the In Vitro Invasion and Migration of Adenocarcinoma Cells, but Not Cell Adhesion

To identify the mechanism by which NE-10 promoted metastasis of LNCaP, we assessed the effect of NE cells on the migration and invasion of LNCaP cells using a Boyden chamber assay. When NE medium was added to the upper chamber with LNCaP cells (Experiment 1), the number of LNCaP cells penetrating through the porous filter without Matrigel coating was significantly greater than that of LNCaP cells in control medium at each given point (Fig. 4A-1). Likewise, when the LNCaP cells were co-cultured with NE-CS cells in the lower chamber (Experiment 2), the number of penetrating LNCaP cells significantly increased (Fig. 4A-2). These results indicated that NE cells enhanced the migration of LNCaP cells. Similarly, the number of LNCaP cells that invaded through the filter with Matrigel significantly increased in the NE medium (Fig. 4B-1, Experiment 1) and in cocultured with NE-CS cells (Fig. 4B-2, Experiment 2), suggesting that NE cells enhanced the invasion of LNCaP cells. Thus, it was speculated that secretions from the NE-CS cells might enhance the migration and in vitro invasion of LNCaP cells. In contrast, the cell adhesion on collagen type IV did not significantly differ between the LNCaP cells with and without NE medium (Fig. 4C). Therefore, the NE-10 allograft promoted pulmonary metastasis of LNCaP cells through enhancement of invasion by facilitating their migration. On the other hand, NE cells had no effect on the proliferation and adhesion to the extracellular matrix of LNCaP cells.

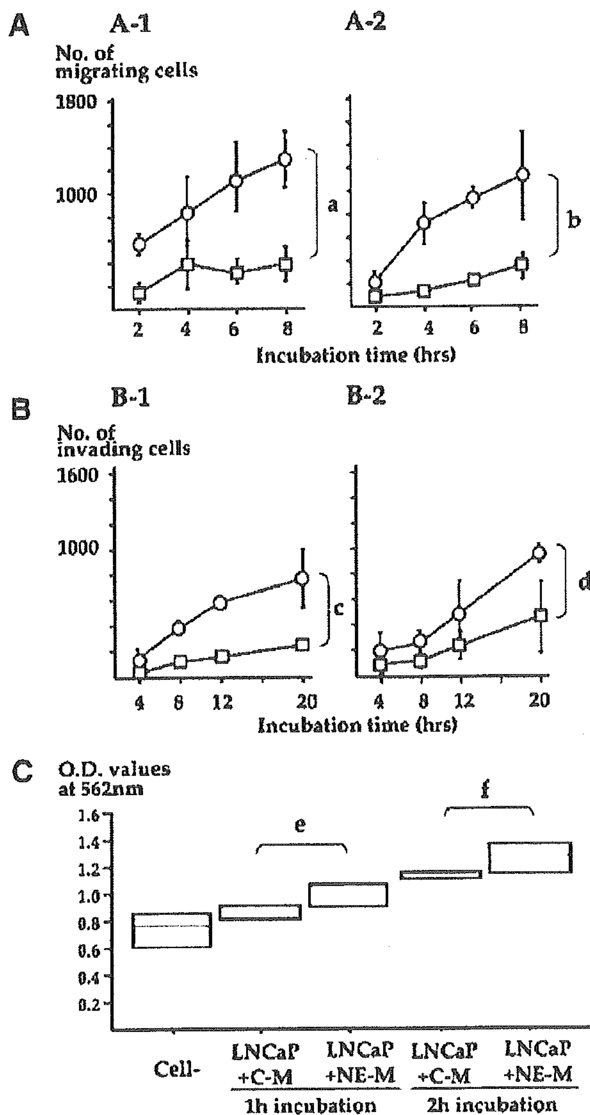

Fig. 4. **A**: Migration of LNCaP cells. Cell migration assay was performed using a porous filter without Matrigel coating. In Experiment 1 (A-1), 1×10^5 of LNCaP cells were placed into the upper chamber of a Boyden chamber with NE medium (open circle) or control medium (open square). In Experiment 2 (A-2), culture medium with (open circle) or without NE-CS cells (open square) was incubated in the lower chamber for 36 hr and then LNCaP cells suspended in culture medium were placed into the upper chamber. The NE-CS cells significantly facilitated the migration of LNCaP cells (a, $P = 0.005$; b, $P < 0.001$; repeated-measures ANOVA). Vertical bars indicate standard deviations of triplicate experiments. **B**: In vitro invasion of LNCaP cells. Invasion assay was performed using a porous filter with Matrigel coating. Protocols of experiments 1 (B-1) and 2 (B-2) were the same as those for the cell migration assay. The NE-CS cells significantly enhanced the invasion of LNCaP cells (c, $P < 0.001$; d, $P < 0.001$; repeated-measures ANOVA). Vertical bars indicate standard deviations of triplicate experiments. **C**: Adhesion of LNCaP cells. LNCaP cells (1×10^3) were seeded onto wells coated with collagen type IV and incubated at 37°C for 1 hr and two hr. NE medium did not significantly stimulate the adhesion of LNCaP cells (e, $P = 0.191$; f, $P = 0.239$; Mann–Whitney *U*-test). Box presents 25th–75th percentiles, as well as median (center line) of triplicate experiments. O.D., optical density; C-M, control medium; NE-M, NE medium.

NE Cells Increase the RNA Expression of Eight Genes of Adenocarcinoma Cells That Include the Actin-Regulating Protein Gelsolin

We examined which genes in LNCaP cells were influenced by treatment with NE medium, using CodeLink™ DNA microarray analysis. The microarray for 10,458 gene profiles detected eight genes with upregulation by NE medium (Table I). Of the eight, we focused on gelsolin since it is an important regulator of actin cytoskeleton dynamics required for cell migration. The expression of mRNA of gelsolin was increased by NE medium 1.9- and 1.5-fold at 4 hr and 8 hr, respectively (Fig. 5A). The increased expression of gelsolin induced by NE medium was confirmed using

TABLE I. Genes Significantly Overexpressed in LNCaP Cells

ACC[a]	Gene name	Expression ratio	
		4 hr	8 hr
AB020716	KIAA0909 Protein	1.6	1.6
NM_000177	Gelsolin	1.9	1.5
NM_003004	Secreted and transmembrane 1 (SECTM1)	2.1	1.6
NM_005224	Dead ringer-like 1 (Drosophila) (DRIL1)	2.0	1.9
NM_005889	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 (APOBEC1), transcript variant 2	1.5	1.8
NM_015196	KIAA0922 protein	1.6	1.5
NM_018321	Hypothetical protein FLJ11100 (FLJ11100)	1.5	1.7
NM_021270	LE hypothetical protein FLJ11100 (FLJ11100) leukocyte-associated IG-like receptor 2 (LAIR2), transcript variant 2	1.9	1.5

Genes with an expression ratio >1.5 were considered to be significantly overexpressed.
[a]GeneBank accession number.

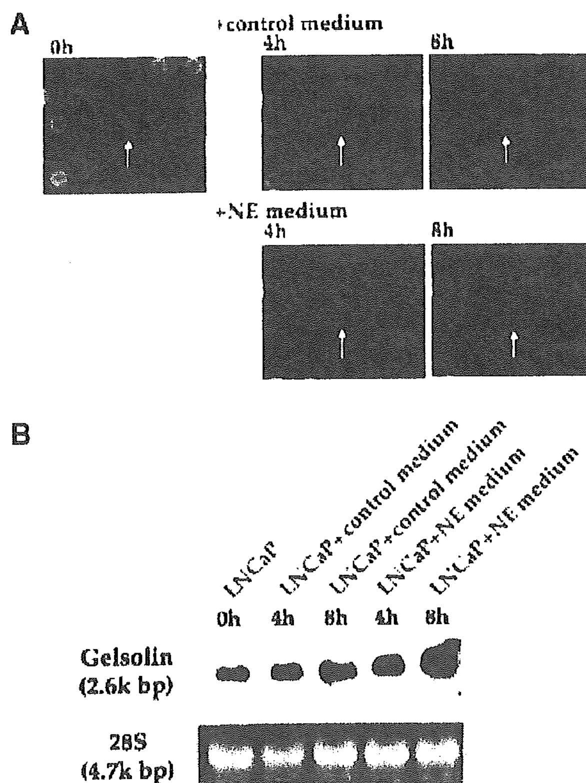

Fig. 5. **A**: Images of DNA microarrays including gelsolin spots in LNCaP cells. The slides show several spots stained with Cy5-streptavidin conjugate (red circles). LNCaP cells with NE medium show prominent signal intensity for gelsolin (arrow) compared to those with control medium at 4 and 8 hr incubation. **B**: Northern blot analysis of mRNA of gelsolin in LNCaP cells. Northern blot analysis demonstrates markedly increased expression of gelsolin in LNCaP cells with NE medium compared to control medium.

Northern blot analysis (Fig. 5B). Thus, it was speculated that the upregulation of gelsolin in LNCaP cells by secretions from NE cells was involved in facilitation of migration of LNCaP cells.

NE Cells Change the Morphology of Adenocarcinoma Cells

We analyzed how NE medium influenced the actin filament dynamics in LNCaP cells. It increased the proportion of LNCaP cells with obvious protrusions (Fig. 6). The conversion of the actin filaments occurred at the bases of protrusions of LNCaP cells. These results suggested that the morphology of LNCaP cells was changed by treatment with NE medium.

DISCUSSION

Although much progress has recently been made in identifying molecular events leading to the development of prostate cancer, the exact mechanisms underlying the acquisition of androgen independence remain poorly understood [13,14]. The possible mechanisms have been reported to be alternations of the activity, function and specificity of AR by mutation or amplification of the *AR* gene [15,16], and activation of intracellular signal transduction pathways that stimulate AR [17,18]. Thus, the mechanism of androgen independence is multifactorial. In addition, recent reports have posited a role of NE differentiated cells in the progression and androgen independence of prostate cancer [9].

NE cells produce growth factors, including vascular endothelial growth factor and transforming growth factor α, which may stimulate growth and accelerate progression of the surrounding adenocarcinoma cells in a paracrine fashion [19]. In addition, NE cells secrete

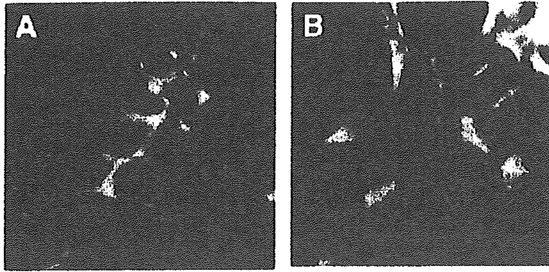

Fig. 6. The LNCaP cells were cultured on fibronectin-coated chamber slides for 6 hr in control medium (**A**) or NE medium (**B**). Filamentous actin was visualized using TRITC-labeled phalloidin under a confocal laser microscope. The LNCaP cells with NE medium possess more prominent protrusions with converting actin filaments at the base than those with control-medium.

a variety of neuropeptides and biogenic amines such as serotonin [1]. It has been clinically reported that the number of NE cells that lack AR increases during androgen ablation therapy [3]. These results indicate that NE cells might play an important role in the development and progression of prostate cancer. However, the prognostic value of NE differentiation is clinically controversial [2,20]. Thus, to investigate the relationship between NE cells and adenocarcinoma cells are mandatory in basic research.

The most critical problem in studying the progression of prostate cancer is the lack of adequate model systems. Although new cell lines and xenografts for human models for NE prostatic carcinoma have been described [21,22], there has been no direct in vivo evidence on whether NE cells influence the development and progression of prostate cancer and androgen independence. Recently, we established the NE-10 allograft and the NE-CS cell line from the ventral prostate of LPB-Tag transgenic mouse line 10 (12T-10) [7,8]. NE-10/NE-CS has the NE property represented by dense core granules in the cytoplasm and androgen-independent growth due to being negative or weakly positive for AR. The development of this NE allograft and NE-CS cell line has provided us with an opportunity to investigate the role of NE cells in prostate cancer.

Previously, we demonstrated that the NE-10 allograft promoted LNCaP tumor growth in castrated mice [9]. In vitro and in vivo studies demonstrated that this effect was mediated by increased AR level/activity in LNCaP cells by NE secretions combined with a low androgen concentration. In the present study, we demonstrated that the NE-10 allograft promoted the metastasis of the LNCaP xenograft in mice having a normal testicular androgen level. The metastatic process consists of multiple steps, including growth at the primary site, invasion into vessels, circulation to the metastatic site, extravasation and growth at distant organs [23]. Cancer cell invasion is a crucial phenomenon for metastasis. It is composed of three steps: cancer cell attachment to the basement membrane, degradation of the extracellular matrix by proteolytic enzymes, and cell migration [24]. We found that the NE-CE cells promoted migration and invasion of LNCaP cells but not cell proliferation and attachment. Thus, it was speculated that the NE cells promoted pulmonary metastasis of LNCaP cells through enhancement of cell invasion by facilitating their migration.

The DNA microarray and Northern blot analysis showed that expression of mRNA of gelsolin in LNCaP cells was increased by the supernatant of NE-CS cells. Gelsolin is an actin-binding protein with well-characterized functions for cytoskeltal reorganization, cell morphology, and motility. Activated gelsolin binds to assembled actin filaments and severs the actin into small fragments (severing). After severing, gelsolin remains attached to the barbed ends of the fragments as a cap to prevent reannealing with each other or elongation at the barbed end (capping). Gelsolin also promotes polymerization of actin monomers to elongate actin filaments (nucleating). Thus, gelsolin mediates the dynamic changes in the actin cytoskeleton for a variety of forms of cell motility [25,26]. In addition, it is known that gelsolin is involved in cellular apoptosis, since it is identified as a substrate of caspase-3 [27]. Gelsolin expression is frequently downregulated in several types of human cancer [28,29], including prostate cancer [30]. Although gelsolin is considered to be a candidate tumor suppressor gene, its role in carcinogenesis remains unclear. Recently, it has been reported that higher expression of gelsolin is associated with a higher risk of recurrence in early stage non-small cell lung cancer and breast cancer [31,32]. In a study on urothelial carcinoma, Rao et al. [33] reported that gelsolin expression decreased in carcinoma in situ and dysplastic lesions compared to benign areas, whereas it gradually increased as the grade and stage became higher. They also demonstrated that higher expression of gelsolin was an independent predictor for recurrence and progression.

In vitro studies have shown that increased expression of gelsolin in cultured fibroblasts results in an increase of cell migration [34,35]. Similarly, it has been demonstrated that overexpression of gelsolin promotes invasion of epithelial cells (MDCK and HEK293T cells) [36]. In the present study, NE cells facilitated the migration of LNCaP cells and increased expression of gelsolin mRNA in LNCaP cells. We also observed that the NE cells enhanced remodeling of the cytoskeletal organization of LNCaP cells. Based upon our evidence and previous studies, it is likely that gelsolin plays an important role in LNCaP tumor cell motility and

invasion, although further study is mandatory to confirm the relationship between upregulation of gelsolin and cell motility in our model.

Nishimura et al. [37] reported that androgen ablation increased the expression of gelsolin in LNCaP cells. The increasing expression of gelsolin enhanced the AR activity under low androgen level, which suggested that gelsolin contributed in maintaining a functional AR signaling pathway even in a low androgen environment. Thus, gelsolin may have a biphasic function in the carcinogenesis and progression of prostate cancer. Expression of gelsolin may be down-regulated in the early stage of carcinogenesis, but may later switch to upregulation and promote metastasis by facilitating tumor cell motility. The surrounding NE cells may contribute to upregulation of mRNA of gelsolin in adenocarcinoma cells as well as a change in the characteristics of adenocarcinoma cells per se by androgen ablation since androgen ablation induces LNCaP cells to transdifferentiate into an NE-like phenotype [38]. Our previous study also demonstrated that NE secretions activated AR of LNCaP cells under low androgen levels [9]. Gelsolin, which is upregulated by ablation of testicular androgens may be involved in the development of hormone-refractory prostate cancer where the cancer can respond to low levels of adrenal androgens.

There are several limitations to this study. The NE-10 allograft and the NE-CS cell line, which were derived from the mouse prostate, are of different origin from human adenocarcinoma cell line LNCaP. The role of human NE cells in human prostate cancer may not be the same as mouse NE cells. In addition, the characteristics of the established cell line, NE-CS could be different from those of the original NE-10 allograft because cells suitable for survival in vitro were selected during establishment of the cell line. Thus, peptides or amines secreted from NE cells may be different in NE-10 allograft and NE-CS cells. There are no ideal human lines for which both in vitro and in vivo NE models are available. In addition, it is unknown which factors secreted from NE cells are involved in increased expression of gelsolin. Because NE-10/NE-CS produces several neuropeptides such as chromogranin A, serotonin, and somatostatin, it will be necessary to investigate if these or other factors are crucial for upregulation of gelsolin in LNCaP cells.

Although we need to generate further evidence about the importance of the interaction between adenocarcinoma cells and NE cells in the field of prostate cancer, our results establish a significant role for NE cells in vivo. Learning how to target NE cells may lead to a breakthrough in controlling progression of prostate cancer to metastasis and becoming hormone refractory to androgen ablation treatment.

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Original Article

Erectile dysfunction following nerve-sparing radical retropubic prostatectomy and its treatment with sildenafil

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Abstract

Background: We retrospectively evaluated the erectile function after nerve-sparing radical retropubic prostatectomy (RRP) and the efficacy of sildenafil for erectile dysfunction (ED) following RRP according to the preoperative erectile function.

Methods: We evaluated 48 Japanese patients who underwent nerve-sparing RRP at the Sapporo Medical University School of Medicine, Sapporo, Japan, between January 1996 and December 2001. Erectile function following nerve-sparing RRP was assessed by a simple mailed questionnaire that was constructed for the study.

Results: Of the 48 patients, 36 had normal erectile function preoperatively, but for 12, function was not sufficient to penetrate. The overall estimated recovery rates of any degree of erection were 50.6% at 36 months and 94.3% at 60 months. However, that of erection sufficient to penetrate was only 17.7% at 36 months and was only seen in bilateral nerve-sparing patients. Sildenafil was effective in 9 of 13 ED patients (69.2%) in both nerve-sparing groups. When patients were divided according to preoperative erectile function, no difference was found in the efficacy rate between patients with normal function and those with ED.

Conclusions: Even bilateral nerve-sparing RRP can not always guarantee a sufficient erection. However, sildenafil is effective for ED following nerve-sparing RRP regardless of the nerve-sparing procedure or preoperative erectile function. Thus, preoperative function alone, although depending on its severity, may not necessarily be a reason for exclusion from receiving nerve-sparing RRP if patients want to have the operation.

Key words erectile dysfunction, nerve-sparing, radical retropubic prostatectomy, sildenafil citrate.

Introduction

It has been a problem that prostate cancer treatments cause patients' quality of life (QOL) to deteriorate.[1–3] Excellent disease control enables long-term survival. Thus, QOL including a better sexual life might be important for patients. Development of surgical procedures, such as the nerve-sparing technique, has enabled many patients to maintain erectile function.[4] However, the procedure does not always guarantee the recovery of function. Sildenafil was reported to be effective for patients with erectile dysfunction (ED) following nerve-sparing radical retropubic prostatectomy (RRP).[5,6] Nerve-sparing RRP can result in the functional recovery of erection and demonstrate the efficacy of sildenafil.

As far as we know, there is no report which considers nerve-sparing-RRP for patients who have ED preoperatively. It has not been clear whether preoperative ED patients can achieve sexual intercourse following nerve-sparing RRP with the help of sildenafil.

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The goal of the present study was to clarify the functional recovery of erectile function following nerve-sparing RRP at Sapporo Medical University School of Medicine, Sapporo, Japan, the efficacy of sildenafil for ED following nerve-sparing-RRP and the rationale of the nerve-sparing procedure and sildenafil treatment for patients with preoperative ED.

Methods

Between January 1996 and December 2001, 66 Japanese patients underwent nerve-sparing RRP for clinically organ-confined prostate cancer at the Sapporo Medical University School of Medicine. The exclusion of 15 patients with neoadjuvant hormone therapy and three with immediate adjuvant hormone therapy left 48 who were retrospectively evaluated in the present study.

Regarding the definition of ED, the National Institutes of Health (NIH) Consensus on ED defines impotence as the consistent inability to attain and maintain a penile erection sufficient to permit satisfactory sexual intercourse.[7] In the present study, we defined normal erectile function as that 'sufficient to penetrate' as defined by NIH and assessed the functional recovery over time after the treatment. Erectile function before surgery was assessed by interview and a questionnaire for erectile function, which was previously validated.[8] We also assessed the status of preoperative erectile function by nocturnal circumferential change with an erectometer.[9] The function after surgery was evaluated by a simple mailed questionnaire newly developed for this study (See Appendix). All patients responded to the questionnaire. The new questionnaire was almost the same as the one for which we previously reported validity without Q4 and Q5, which were added to evaluate the time the patient recovered from ED.[8] We assessed the recovery rate according to the quality of erection, which was categorized into any degree of erection and erection sufficient to penetrate.

Basically, the nerve-sparing procedure is considered based on subjective and objective erectile function corresponding to age, cancer characteristics (including PSA and the Gleason's sum of the biopsy specimen), cancer location estimated from a positive core and surgical findings. However, when patients with ED strongly hoped for a nerve-sparing procedure before surgery, this was also considered.

Pretreatment serum prostate-specific antigen (PSA) concentrations were measured by radioimmunoassay (Hybritech, San Diego, CA). Clinical stages were classified based on the TNM classification of the 1997 American Joint Committee on Cancer (AJCC).[10] Biochemical failure was defined by detectable serum PSA (0.2 ng/mL or greater) and the day of recurrence was the first day of PSA detection.[11] We included data on erectile function before recurrence in the present study.

The efficacy of sildenafil was evaluated in 13 patients who complained of ED following nerve-sparing RRP and wanted to receive treatment for the disease. They consisted of nine patients with preoperative normal erectile function and four patients with preoperative ED. Achievement of sufficient rigidity for sexual intercourse was defined as 'effective'. We initiated sildenafil treatment with a dose of 25 mg and increased to 100 mg, depending on efficacy, if necessary. We used the computer program StatView 5.0 for Windows (SAS Institute, Cary, NC) for statistical analyses. The recovery rate of erectile function was determined by the Kaplan-Meier method with a log-rank test for statistical analysis. The Mann–Whitney *U*-test and Fisher's exact test were used for comparison of characteristics between two groups. A *P*-value of <0.05 was considered to be statistically significant.

Results

The characteristics of the patients in the current study are shown in Table 1. Through the assessment for erectile function before surgery, 36 patients were considered to have normal erectile function and 12 ED. Of the 36 with normal function, bilateral nerve-sparing was done for 27 (75.0%) and unilateral nerve-sparing for 9 (25.0%). Of the 12 patients with ED, bilateral nerve-sparing was done for 4 (33.3%) and unilateral nerve-sparing for 8 (66.7%).

In a pathological study of surgical specimens, pT3 disease was found in eight patients (17%). Biochemical failure was seen in 10 patients (seven of 40 in pT2, three of eight in pT3). Salvage radiotherapy was performed for six, androgen ablation for two and watchful-waiting for two patients. There was no death due to prostate cancer. The single significantly different feature was age between those with and without preoperative ED (Mann–Whitney *U*-test; $P = 0.016$).

Nocturnal penile circumferential change was assessed for 11 patients in the preoperative ED group and 35 patients in the preoperative group (Table 1). The median maximal penile circumferential changes were 15 mm and 25 mm, respectively ($P = 0.008$; Mann–Whitney *U*-test). Unfortunately, the cause of ED in this preoperative ED group had not been assessed and was unclear; however, it was considered to be organic and related to higher age and vascular status.