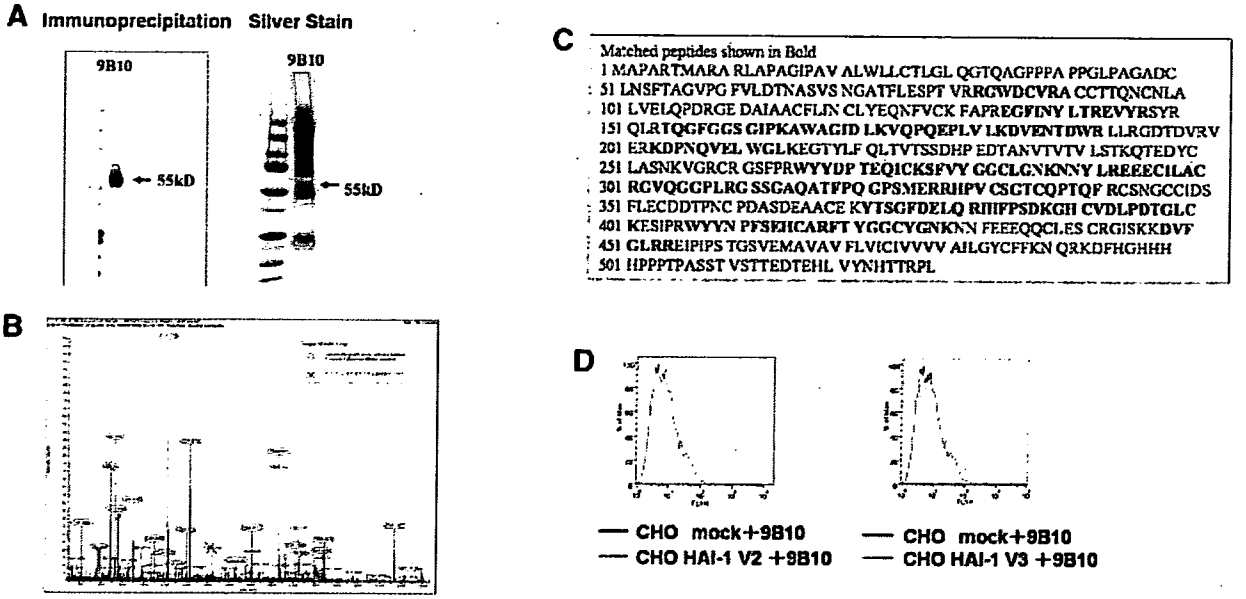


Fig. 5. Identification of mAb 9B10 antigen. **A**: immunoprecipitation with mAb 9B10. The band that appeared at 55 kDa (indicated by an arrow) was excised from the gel and analyzed by mass spectrometry. **B**: Encircled high-intensity spectra circle indicate the peptide, the sequence of which corresponded to the amino acid sequence of human HAI-1. **C**: Boldface indicates the sequence of the detected peptide. **D**: Flow cytometry of the reactivity of mAb 9B10 with CHO cells transfected with cDNA of each subunit of HAI-1. mAb9B10 reacted with CHO cells transfected with cDNA of each subunit of HAI-1.

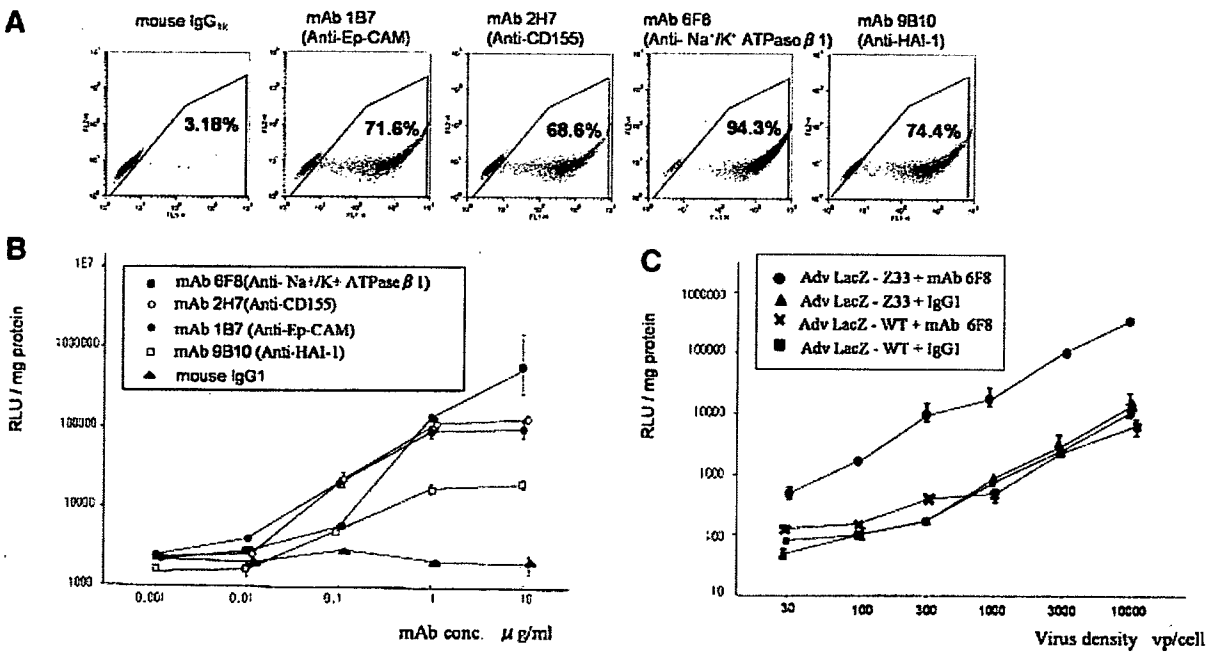


Fig. 6. Transfection efficiency into PC-3 cells with each fiber mutant adenovirus-mediated mAb. **A**: Numbers presented in each panel indicate the percentage of cells expressing EGFP. Cells transfected using AdvEGFP-FZ33 together with mIgG1 showed low expression of EGFP. Cells transfected using AdvEGFP-FZ33 together with mAbs 1B7(anti-Ep-CAM mAb), 2H7(anti-CD155 mAb), 6F8(Na,K-ATPase β1 mAb), and 9B10(anti-HAI-1 mAb) showed enhanced expression of EGFP. **B**: The cells were lysed, and assayed for β-Gal activity using a commercial kit (n = 4). AdvEGFP-FZ33 together with mAbs 1B7, 2H7, 6F8, and 9B10 showed high transduction efficiency compare with control IgG. **C**: Adv LacZ - FZ33 together with mAb 6F8 showed high transduction efficiency compared with wild type adenovirus (Adv Lacz–WT).

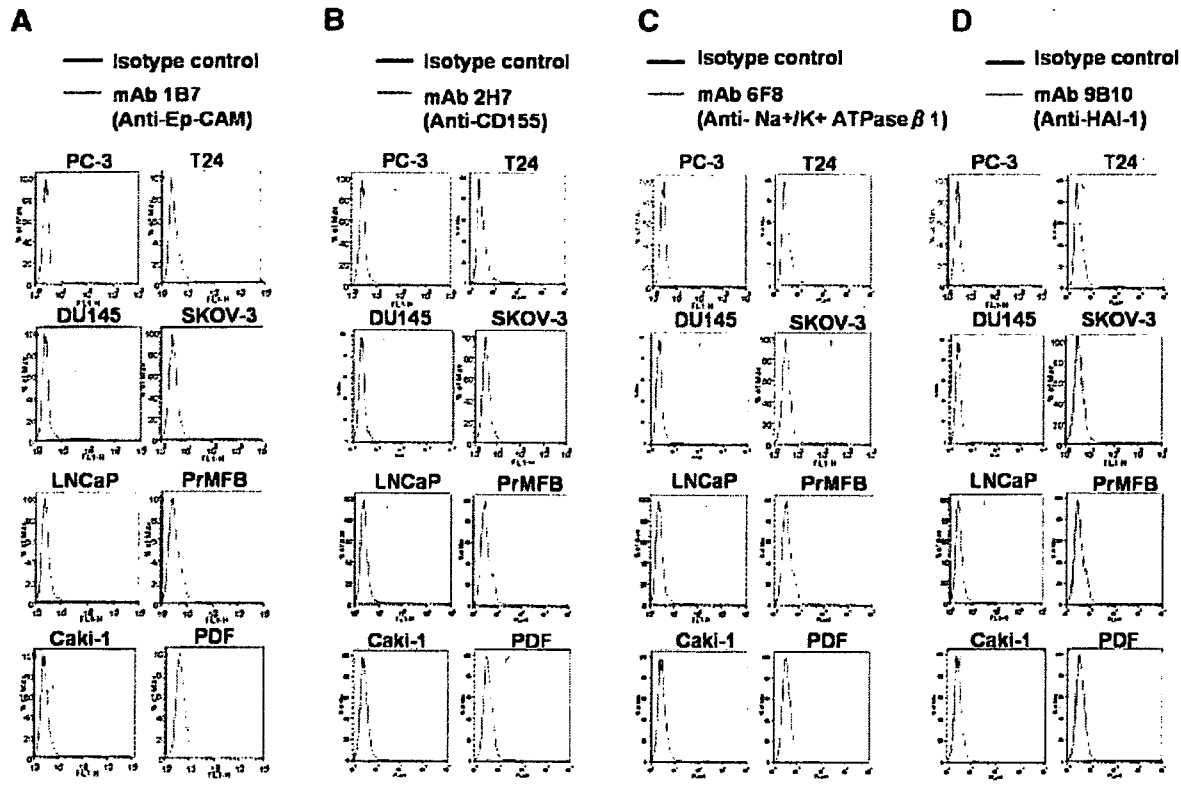


Fig. 7. Reactivities of established mAbs with various human cell lines. **A**: Antigen of mAb 1B7 was expressed on the surface of PC-3, DU145, LNCaP, Caki-1, T24, and SKOV-3 but not PrMFB and PDF. **B**,**C**: Antigens of mAbs 1B7 and 6F8 were expressed on the surface of each cell line. **D**: Antigen of mAb 9B10 was expressed on the surface of PC-3, DU145, LNCaP, Caki-1, and T24 but not SKOV-3, PrMFB, or PDF.

DISCUSSION

Volspers et al. [6] constructed a Z33-modified adenovirus vector and reported that the transduction efficiency of this modified vector in epidermal growth factor receptor (EGFR)-expressing cells was strongly and dose-dependently enhanced by combination with an EGFR-specific monoclonal antibody. They suggested that antibody-mediated targeting of the Z33-modified adenovirus vector could be applied for directed gene transfer to a wide variety of cell types by simply changing the target-specific antibody. Tanaka et al. [8] evaluated, both in vitro and in vivo, the extent of retargeting toward and therapeutic effectiveness against carcinoembryonic antigen (CEA)-positive gastric cancers when using the fully human CEA antibody complex with Adv-FZ33. They generated Ax3CAUP-FZ33 (UP-FZ33), an Adv-FZ33 derivative vector expressing a therapeutic gene (*Escherichia coli* uracil phosphoribosyltransferase) that converted 5-fluorouracil (5-FU) directly to 5-fluoro-UMP. UP-FZ33 with the anti-CEA mAb enhanced the cytotoxicity of 5-FU by 10.5-fold in terms of the IC_{50} against a CEA-positive gastric cancer cell line compared with control IgG4. In a nude mouse peritoneal dissemination model, tumor growth in mice treated by UP-FZ33 premixed with the anti-CEA mAb was significantly suppressed, and the median survival time was significantly longer than in the control group.

Surface antigens may be a viable target for antibody-mediated gene therapy. Although, PSA is a clinically important biomarker in prostate cancer [10,11], PSA is not a surface antigen [12]. Although, prostate-specific membrane antigen (PSMA) [13] is a type II membrane antigen, the PSMA expression pattern is not fully restricted to the prostate [14,15]. Therefore, we investigated target molecules amenable to gene therapy with Adv-FZ33. In this study, we immunized mice with three human cell lines that were androgen-independent (PC-3 and DU145) or androgen-dependent (LNCaP) [16], for generating various types of mouse mAbs. We have performed to screening the target antibodies using human prostate cancer cell line PC-3. Although, our Adv-FZ33 has intact CAR-binding structure and retains CAR-binding ability, CAR protein is downregulated in the highly tumorigenic PC-3 cell line [17] and the transduction efficiency of adenovirus is quit low (Fig. 6). Furthermore, PC-3 does

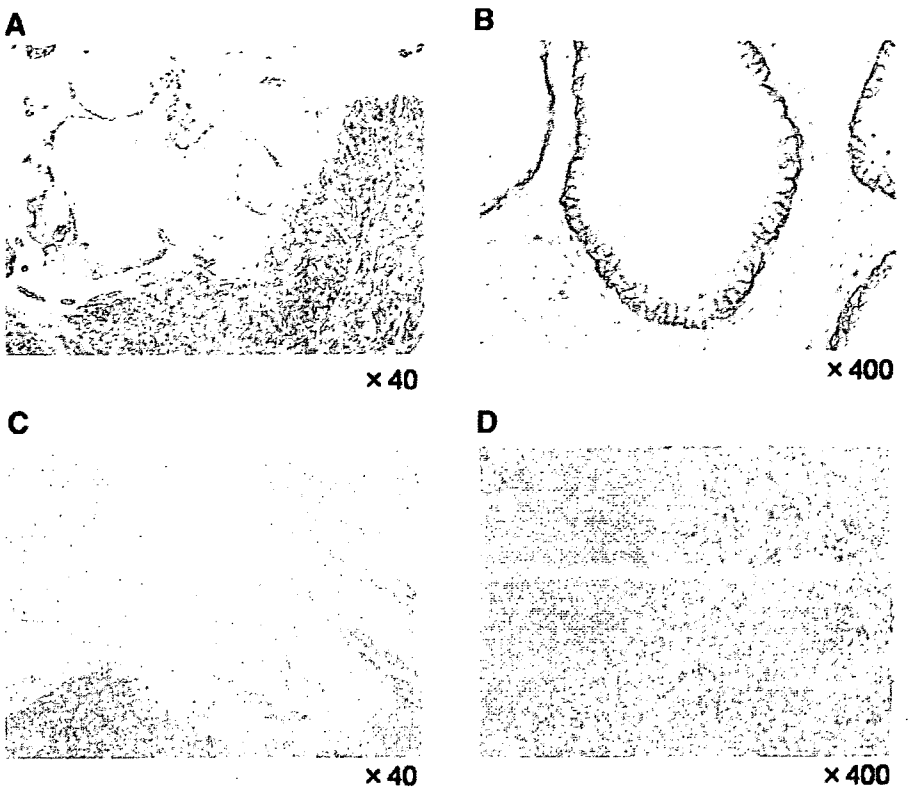


Fig. 8. Immunohistochemical findings for prostate cancer cells and normal epithelial cells of the prostate. **A**: Prostate cancer and normal epithelial cells shows strong immunoreactivity for mAb 1B7. **B**: Basolateral cell surface of normal epithelium shows strong immunoreactivity for mAb 1B7. **C**: Prostate cancer shows strong immunoreactivity for mAb 1B7 but normal epithelial cells do not. **D**: Normal epithelial cells are not stained. Stromal cells are not stained in each sample.

not express PSMA. The first aim of this study is to target adenovirus to resistant cell line PC-3.

We purified four different mAbs and confirmed high transduction efficiency by flow cytometry and chemiluminescent β-Gal reporter gene assay. The target molecules of mAbs 1B7, 2H7, 6F8, and 9B10 were Ep-CAM, CD155, Na,K-ATPase β1, and HAI-1, respectively. CD155 and Na,K-ATPase β1 found to be widely expressed by flow cytometric analysis are inappropriate molecules for tumor targeting. The anti-Ep-CAM antibody mAb 1B7 and anti-HAI-1 antibody mAb 2H7 showed reactivity with cancer cell lines other than fibroblast cell lines. We could not find reactivity with prostate cancer samples for mAb 2H7, 6F8, or 9B10. These mAbs may be unsuitable for staining of samples fixed in formalin. In mAb 1B7, we found that Ep-CAM expression on prostate cancer cells was stronger than on normal epithelial cells, but not significantly. In 1979, Ep-CAM was discovered in a search for novel cell surface antigens expressed on neoplastic tissue [18]. Ep-CAM is known to be expressed on the basolateral cell surfaces of selected normal epithelia and many carcinomas [19–22]. Of particular interest, overexpression of Ep-CAM has been reported in prostate cancer [21,23]. Clinical trials of mAbs directed against Ep-CAM for immunologic therapy have been conducted in patients with colon cancer [24]. Recently, clinical phase I study with anti-Ep-CAM humanized IgG1 have been performed in patients with hormone refractory prostate cancer by Oberneder et al. [25]. Heideman et al. [26] showed that Ep-CAM targeted vectors using bispecific antibodies against the adenovirus fiber-knob protein, and Ep-CAM may be useful for gastric and esophageal cancer-specific gene therapy. HAI-1 is a novel Kunitz-type serine protease inhibitor first reported in 1997 [27]. Although, HAI-1 is broadly expressed in epithelial cells of most human tissues [28,29], Knudsen et al. [30] reported that its expression was significantly increased in localized prostate cancer and was present in most prostate cancer metastases compared to normal prostate glands. Furthermore, HAI-1 overexpression in prostate cancer was predictive of prostate-specific antigen recurrence. Nagakawa et al. [31] demonstrated that significantly increased serum levels of HAI-1 were detected in patients with prostate cancer, indicating that HAI-1

would be a potential tumor marker for prostate cancer. Ep-CAM and HAI-1 overexpressed in prostate cancer may be potential targets for prostate cancer gene therapy with Adv-FZ33, although therapeutic effectiveness must be evaluated as well. In future clinical application, we would like to use Adv-FZ33 premixed with prostate cancer targeting mAb prior to injection. This method seems to be more practical for administration systemically.

In addition to mAb 1B7 and 9B10, we may be able to establish new prostate cancer-specific mAbs through the hybridoma screening system that was designed in this study. Previously, Lampe et al. [32] performed fusions 25 times and developed three mAb directed against prostate associated antigens that might identify potential new therapeutic targets through screening of circa 25,000–50,000 hybridomas. We evaluated only 2,500 wells through three fusions and identified potential four target molecules. Overall, this approach of inductive method using FZ33 fiber-modified adenovirus is reliable strategy for screening useful mAbs that recognize target molecules in prostate cancer gene therapy as well as antibody therapy and diagnosis.

CONCLUSIONS

We established hybridoma from mice immunized with prostate cancer cell lines and selected anti-Ep-CAM mAb and anti-HAI-1 mAbs. Using Adv-FZ33, these mAbs increased transduction efficiency to prostate cancer cells. Gene transduction via Ep-CAM and HAI-1 may be a novel strategy for treatment of prostate cancer.

ACKNOWLEDGMENTS

The authors thank Ms. Toshie Kurohata for her technical assistance. This study was partly supported by the Stiftelsen Japanese-Swedish Cooperative Foundation (T. Tsukamoto), by Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan (K. Kato and H. Hamada) and by Grant-in-Aid for Scientific Research on Priority Areas "Cancer" from the Ministry of Education, Culture, Sports, Science and Technology (K. Kato, K. Nakamura, and H. Hamada).

REFERENCES

1. Jemal A, Thomas A, Murray T, Thun M. Cancer statistics. CA Cancer J Clin 2002;52:23–47.
2. Kasper S, Cookson MS. Mechanism leading to the development of hormone-resistant prostate cancer. Urol Clin N Am 2006;33: 201–210.
3. Macrae EJ, Giannoudis A, Ryan R, Brown NJ, Hamdy FC, Maitland N, Lewis CE. Gene therapy for prostate cancer: Current strategies and new cell-based approaches. Prostate 2006;66:470–494.
4. Brody SL, Crystal RG. Adenovirus-mediated in vivo gene transfer. Ann N Y Acad Sci 1994;716:90–101; (discussion 101–103).
5. Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 1997;275:1320–1323.
6. Volpers C, Thirion C, Biermann V, Hussmann S, Kewes H, Dunant P, von der Mark H, Herrmann A, Kochanek S, Lochmuller H. Antibody-mediated targeting of an adenovirus vector modified to contain a synthetic immunoglobulin g-binding domain in the capsid. J Virol 2003;77:2093–2104.
7. Hisataki T, Itoh N, Suzuki K, Takahashi A, Masumori N, Tohse N, Ohmori Y, Yamada S, Tsukamoto T. Modulation of phenotype of human prostatic stromal cells by transforming growth factor-betas. Prostate 2004;58:174–182.
8. Tanaka T, Huang J, Hirai S, Kuroki M, Kuroki M, Watanabe N, Tomihara K, Kato K, Hamada H. Carcinoembryonic antigen-targeted selective gene therapy for gastric cancer through FZ33 fiber-modified adenovirus vectors. Clin Cancer Res 2006;12: 3803–3813.
9. Kitamura H, Torigoe T, Asanuma H, Hisasue SI, Suzuki K, Tsukamoto T, Satoh M, Sato N. Cytosolic overexpression of p62 sequestosome 1 in neoplastic prostate tissue. Histopathology 2006;48:157–161.
10. Getzenberg RH, Abrahamsson PA, Canto EI, Chinnaiyan AM, Djavan B, Laxman B, Ogawa O, Slawin K, Tomlins SA, Yu J. Advances in biomarkers for prostatic disease. In: McConnell J, Denis L, Akaza H, Khoury S, Schalken J, editors. Prostate Cancer. Paris: Health Publications; 2006. pp 85–148.
11. Lieberman R. Evidence-based medical perspectives: The evolving role of PSA for early detection, monitoring of treatment response, and as a surrogate end point of efficacy for interventions in men with different clinical risk states for the prevention and progression of prostate cancer. Am J Ther 2004;11:501–506.
12. Migita T, Oda Y, Naito S, Morikawa W, Kuwano M, Tsuneyoshi M. The accumulation of angiostatin-like fragments in human prostate carcinoma. Clin Cancer Res 2001;7:2750–2756.
13. Horoszewicz JS, Kawinski E, Murphy GP. Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and serum of prostatic cancer patients. Anticancer Res 1987;7:927–935.
14. Israeli RS, Powell CT, Corr JG, Fair WR, Heston WD. Expression of the prostate-specific membrane antigen. Cancer Res 1994;54: 1807–1811.
15. Sokoloff RL, Norton KC, Gasior CL, Marker KM, Grauer LS. A dual-monoclonal sandwich assay for prostate-specific membrane antigen: Levels in tissues, seminal fluid and urine. Prostate 2000;43:150–157.
16. Janssen T, Darro F, Petein M, Raviv G, Pasteels JL, Kiss R, Schulman CC. In vitro characterization of prolactin-induced effects on proliferation in the neoplastic LNCaP, DU145, and PC-3 models of the human prostate. Cancer 1996;77:144–149.
17. Okegawa T, Li Y, Pong RC, Bergelson JM, Zhou J, Hsieh JT. The dual impact of coxsackie and adenovirus receptor expression on human prostate cancer gene therapy. Cancer Res 2000;60:5031–5036.
18. Herlyn M, Steplewski Z, Herlyn D, Koprowski H. Colorectal carcinoma-specific antigen: Detection by means of monoclonal antibodies. Proc Natl Acad Sci USA 1979;76:1438–1442.
19. Momburg F, Moldenhauer G, Hammerling GJ, Moller P. Immunohistochemical study of the expression of a Mr 34,000

human epithelium-specific surface glycoprotein in normal and malignant tissues. Cancer Res 1987;47:2883–2891.

20. Xie X, Wang CY, Cao YX, Wang W, Zhuang R, Chen LH, Dang NN, Fang L, Jin BQ. Expression pattern of epithelial cell adhesion molecule on normal and malignant colon tissues. World J Gastroenterol 2005;11:344–347.
21. Went P, Vasei M, Bubendorf L, Terracciano L, Tornillo L, Riede U, Kononen J, Simon R, Sauter G, Baeuerle PA. Frequent high-level expression of the immunotherapeutic target Ep-CAM in colon, stomach, prostate and lung cancers. Br J Cancer 2006;94: 128–135.
22. Heinzelmann-Schwarz VA, Gardiner-Garden M, Henshall SM, Scurry J, Scolyer RA, Davies MJ, Heinzelmann M, Kalish LH, Bali A, Kench JG, Edwards LS, Vanden Bergh PM, Hacker NF, Sutherland RL, O'Brien PM. Overexpression of the cell adhesion molecules DDR1, claudin 3, and Ep-CAM in metaplastic ovarian epithelium and ovarian cancer. Clin Cancer Res 2004;10:4427–4436.
23. Poczatek RB, Myers RB, Manne U, Oelschlager DK, Weiss HL, Bostwick DG, Grizzle WE. Ep-Cam levels in prostatic adenocarcinoma and prostatic intraepithelial neoplasia. J Urol 1999; 162:1462–1466.
24. Mosolits S, Nilsson B, Mellstedt H. Towards therapeutic vaccines for colorectal carcinoma: A review of clinical trials. Expert Rev Vaccines 2005;4:329–350.
25. Oberneder R, Weckermann D, Ebner B, Quadt C, Kirchinger P, Raum T, Locher M, Prang N, Baeuerle PA, Leo E. A phase I study with adecatumumab, a human antibody directed against epithelial cell adhesion molecule, in hormone refractory prostate cancer patients. Eur J Cancer 2006;42:2530–2538.
26. Heideman DA, Snijders PJ, Craanen ME, Bloemena E, Meijer CJ, Meuwissen SG, van Beusechem VW, Pinedo HM, Curiel DT, Haisma HJ, Gerritsen WR. Selective gene delivery toward gastric and esophageal adenocarcinoma cells via EpCAM-targeted adenoviral vectors. Cancer Gene Ther 2001;8:342–351.
27. Shimomura T, Denda K, Kitamura A, Kawaguchi T, Kito M, Kondo J, Kagaya S, Qin L, Takata H, Miyazawa K, Kitamura N. Hepatocyte growth factor activator inhibitor, a novel Kunitz-type serine protease inhibitor. J Biol Chem 1997;272:6370–6376.
28. Kataoka H, Suganuma T, Shimomura T, Itoh H, Kitamura N, Nabeshima K, Koono M. Distribution of hepatocyte growth factor activator inhibitor type 1 (HAI-1) in human tissues. Cellular surface localization of HAI-1 in simple columnar epithelium and its modulated expression in injured and regenerative tissues. J Histochem Cytochem 1999;47:673–682.
29. Oberst M, Anders J, Xie B, Singh B, Ossandon M, Johnson M, Dickson RB, Lin CY. Matriptase and HAI-1 are expressed by normal and malignant epithelial cells in vitro and in vivo. Am J Pathol 2001;158:1301–1311.
30. Knudsen BS, Lucas JM, Fazli L, Hawley S, Falcon S, Coleman IM, Martin DB, Xu C, True LD, Gleave ME, Nelson PS, Ayala GE. Regulation of hepatocyte activator inhibitor-1 expression by androgen and oncogenic transformation in the prostate. Am J Pathol 2005;167:255–266.
31. Nagakawa O, Yamagishi T, Akashi T, Nagaike K, Fuse H. Serum hepatocyte growth factor activator inhibitor type I (HAI-I) and type 2 (HAI-2) in prostate cancer. Prostate 2006;66:447–452.
32. Lampe MI, Molkenboer-Kuenen JD, Oosterwijk E. Development of new prostate specific monoclonal antibodies. Prostate 2004; 58:225–231.

Increased Akt and phosphorylated Akt expression are associated with malignant biological features of prostate cancer in Japanese men

Yousuke Shimizu, Takehiko Segawa, Takahiro Inoue, Taizo Shiraishi*, Toru Yoshida, Yoshinobu Toda†, Tomomi Yamada‡, Naoko Kinukawa‡, Naoki Terada, Takashi Kobayashi, Hidefumi Kinoshita§, Toshiyuki Kamoto, Eijiro Nakamura and Osamu Ogawa

*Department of Urology, Kyoto University Graduate School of Medicine, *Department of Secondary Pathology, Mie University School of Medicine, †Anatomical Center for Kyoto University Graduate School of Medicine, ‡Division of Medical Infomatics, Kyushu University Hospital, and §Department of Urology, Kansai Medical University, Japan*

Accepted for publication 2 March 2007

OBJECTIVE

To investigate the relationship between the expression of Akt (a serine/threonine kinase that plays a central role in tumorigenesis), phosphorylated Akt (p-Akt), prostate cancer tumour grade, androgen receptor (AR)-staining score, and Ki67 labelling index (LI) in Japanese men.

PATIENTS, MATERIALS AND METHODS

The expression and activation of the cell survival protein Akt was analysed by immunohistochemical staining of paraffin-embedded tissue microarray sections of prostate carcinoma taken from 52 Japanese men who under radical prostatectomy. The correlation between the expression of Akt and p-Akt, and their relationship to primary Gleason grade, AR expression and Ki-67 LI was investigated.

RESULTS

The expression of Akt and p-Akt were positively related to primary Gleason grade (Fisher's exact test, $P = 0.002$ and $P = 0.032$, respectively). Both AR-staining score and Ki67 LI were were positively related to the expression of Akt (both $P < 0.001$) and p-Akt ($P < 0.001$ and $P = 0.008$, respectively). There was a significant positive correlation between the expression of Akt and p-Akt (Spearman's correlation, $r = 0.644$, $P < 0.001$).

CONCLUSIONS

Increased expression of both Akt and p-Akt were associated with higher tumour grade as well as a higher AR-staining score and Ki67 LI. These data indicate that Akt and p-Akt might be molecular markers for detecting malignant biological features of prostate cancer in the Japanese population.

KEYWORDS

prostate cancer, Akt, phosphorylated Akt, androgen receptor

INTRODUCTION

Prostate cancer is the commonest visceral malignancy and the second leading cause of cancer mortality in men in Western countries [1]. Prostate cancer is initially dependent on androgens for growth. Most tumours are primarily treated with surgery or radiation therapy, but disease that is in an advanced stage at diagnosis and disease that progresses after primary treatment are managed with androgen-ablation therapy (AAT) [2]. Unfortunately, most tumours treated with AAT alone become resistant to AAT [3]. When this occurs, the prognosis is poor because a standard therapy has yet to be established for this group. Thus, there is an urgent need to improve our understanding of the biology of prostate cancer and to establish new prognostic markers to aid in the selection of unfavourable prognostic groups for whom adjuvant therapy is indicated.

In 1997, the tumour-suppressor gene, PTEN was identified as a gene that is mutated in multiple sporadic tumour types [4,5]. In prostate cancer cell lines (PC3 and LNCaP) a deletion or point mutation of PTEN have been reported [4,5]. PTEN is a lipid phosphatase that negatively regulates the phosphatidylinositol 3-kinase (PI3K) signalling pathway [6]. This pathway is important for cell proliferation and cell survival, and PTEN antagonizes the activity of the PI3K oncoproteins, as it functions as a tumour suppressor. Thus, the PTEN-PI3K-signalling pathway functions as a crucial regulator of cell survival decisions. Important downstream targets of the PTEN-PI3K signalling pathway include the serine-threonine protein kinase B family, or Akt. Members of this family, Akt-1, Akt-2 and Akt-3, are broadly expressed in most organs and tissues. The relative contribution of specific Akt isoforms to the biological activities regulated by them was unknown until recently, but the development of 'knockout' mice for each of the isoforms, as well as isoform-specific small interfering RNAs for analysis in cultured cells, have revealed the specific functions of individual Akt proteins. Briefly, a targeted disruption of Akt-1 in mice has shown its role in the regulation of body size and adipogenesis [7]. As for Akt-2, disruption of this isoform in mice leads to severe insulin resistance and diabetes [8], and for Akt-3, mice lacking in Akt-3 had in 20%

© 2007 THE AUTHORS
JOURNAL COMPILATION © 2007 BJU INTERNATIONAL

decrease in brain size [9]. The net result of the activation of all the Akt isoforms is protection from apoptosis and an increase in cell proliferation. Accumulating evidence suggests that the activation of this key survival kinase plays an important role in tumour development. In a transgenic mouse model (murine prostate Akt kinase transgenic, MPAKT) a constitutively active Akt was overexpressed in mouse prostate epithelia, which resulted in prostate intraepithelial neoplasia [10]. Prostate cancer cell line studies have shown that reduced apoptotic response, release from the cell-cycle block, and regulation of androgen receptor (AR) expression can be triggered by overexpression and activation of Akt [11–13]. Immunohistochemical studies of Akt expression in human prostate cancer tissues showed that Akt is upregulated in prostate cancer and that expression was correlated with tumour progression in Western populations [14–17]. Recently, Le Page *et al.* [18] examined the expression and localization of the three Akt isoforms, suggesting a particular role of Akt-1 expression as a prognostic marker depending on its localization (cytoplasmic or nuclear).

Prostate cancer incidence and mortality rates have been rising over the last two decades, but remain lower in Asian countries, including Japan, than in Western countries. The difference might be partly due to genetic factors, such as ras mutations [19] and polymorphism of the AR and enzymes that participate in androgen metabolism, as well as dietary or environmental factors [20]. Hence, investigating the risk factors for disease progression in Japanese men with prostate cancer is important for advancing our understanding of the disease. In the present study, prostate carcinoma tissue taken from Japanese men at RP was analysed for the expression of Akt and phosphorylated Akt (p-Akt), and their relationships with tumour grade, AR-staining score, Ki67 labelling index (LI), and PSA-defined disease-free survival rate were evaluated.

PATIENTS, MATERIALS AND METHODS

Prostate tissue specimens were obtained from 52 consenting patients who had radical prostatectomy (RP) for prostate cancer between June 1997 and August 2001 at Kyoto University Hospital, Japan. None of the patients had a diagnosis of metastatic cancer or had received hormonal therapy before RP.

Variable	Median (range) or N
Age, years	68 (50–76)
PSA level before RP, ng/ml	9.9 (3.8–120)
Follow-up, months	81.6 (19.2–106.9)
Pathological T stage	
pT2	15
pT3	37
Gleason sum	
5	2
6	2
7	45
8	0
9	3
10	0
Primary Gleason score	
3	39
4	12
5	1
Surgical margin	
negative	17
positive	35

TABLE 1 The demographic and clinicopathological features of the 52 patients

All clinical and pathological data were obtained from medical records and are summarized in Table 1. The date of PSA failure was considered to be when the first blood sample after RP yielded a detectable PSA level (≥0.1 mg/mL). When serum PSA did not decline to <0.1 ng/mL after RP, the date of failure was defined as the date of RP. The patients were followed until 31 March 2006, for a median (range) of 81.6 (19.2 to 106.9) months. The tissue microarrays (TMAs) were constructed as previously described [21]. Three cores from tumour-bearing tissue were obtained from each patient, giving a total of 156 cancer spots.

Standard indirect immunoperoxidase procedures were used on the paraffin-embedded sections. Anti-AKT1/2 polyclonal antibody (N-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA), antip-Akt (Ser473) monoclonal antibody (Cell Signalling Technology, Beverly, MA, USA), anti-AR monoclonal antibody (2F12 Novocastra, Newcastle, UK), and anti-Ki67 monoclonal antibody (MIB-1, DAKO, Kyoto, Japan) were used as primary antibodies at dilutions of 1 : 400, 1 : 100, 1 : 100, and 1 : 100, respectively. The chromogen was 3,3'-diaminobenzidine tetrahydrochloride. Tumours with known positivity were used as positive controls for all antibodies. For the negative controls, primary antibodies were omitted.

The results of Akt and p-Akt staining were interpretation by two of the authors (T.I. and Y.S.) 'blinded' to the clinicopathological data and all spots in the arrays were examined. For Akt and p-Akt, total staining was scored as the product of the staining intensity (on a scale of 0–3) and the percentage of cells stained, resulting in a scale of 0–300 [16]. Staining intensity in the cytoplasm and plasma membrane was defined as follows: no cells stained positively, 0; weak staining, 1; moderate staining, 2; and strong staining, 3; as previously described [16]. Nuclear staining was considered representative of Ki67, while both cytoplasmic and nuclear staining of the epithelium was considered positive for AR. The Ki67 LI was defined as the percentage of the nuclear area stained. The AR-staining score was defined as follows: weak AR staining, 1; moderate , 2; and strong, 3; as previously described [21]. To evaluate the prognostic value of each marker, the mean staining score of Akt, p-Akt and AR (the sum of the score of the three cancer spots in each case divided by three) and the mean Ki67 LI (the sum of the LIs of the three cancer spots in each case divided by three) were calculated for each case. Expression of Akt, p-Akt, the Ki67 LI, and AR-staining score were dichotomized according to a threshold of 140, 140, 5, and 1.6, respectively.

Spearman's rank correlation coefficient was used to analyse the correlation between Akt

© 2007 THE AUTHORS
JOURNAL COMPILATION © 2007 BJU INTERNATIONAL

FIG. 1. Representative immunohistochemical staining of Akt, p-Akt, AR, and Ki67 expression in human prostatic adenocarcinoma. (A, C, E and G, Gleason grade 4; B, D, F and H, Gleason grade 3). (A) Immunohistochemical staining with an Akt antibody showing strong cytoplasmic staining; this would be given a score of 270 [90% of the cells × 3 (strong staining)]. (B) Immunohistochemical staining with an Akt antibody showing no staining; this would be given a score of 0. (C) Immunohistochemical staining with a p-Akt antibody showing strong cytoplasmic staining; this would be given a score of 285 [95% of the cells × 3 (strong staining)]. (D) Immunohistochemical staining with a p-Akt antibody showing no staining; this would be given a score of 0. (E) Immunohistochemical staining with an AR antibody showing strong cytoplasmic and nuclear staining; this would be given a score of 3. (F) Immunohistochemical staining with an AR antibody showing no staining; this would be given a score of 0. (G) Immunohistochemical staining with a Ki67 antibody showing strong nuclear staining; the Ki67 LI was 12. (H) Immunohistochemical staining with a Ki67 antibody showing no staining; the Ki67 LI was 0.

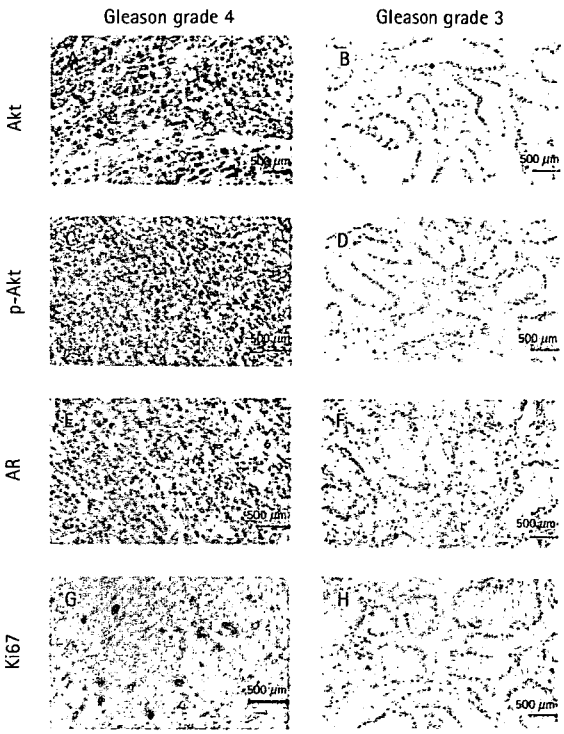
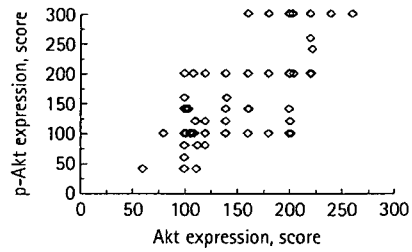


FIG. 2. Scatterplot of Akt and p-Akt expression scores in each cancer spot. Akt expression correlates with that of p-Akt. Spearman's correlation, r = 0.644; P < 0.001.



and p-Akt staining scores in each of the 156 cancer spots. The chi-squared test or Fisher's exact probability test were used to analyse the relationship between the staining score of Akt or p-Akt and the AR-staining score, the Ki67 LI or primary Gleason grade in each cancer spot. Cox proportional-hazards model was used for univariate and multivariate survival analyses to evaluate the risk factors associated with PSA failure. To obtain a multivariate model with the statistically significant variables, a stepwise-selection procedure was used. Categorical variables included age, preoperative PSA level, stage, Gleason sum, primary Gleason score, TMA Gleason sum, TMA primary Gleason score, surgical margin, the Ki67 LI and the staining score of Akt, p-Akt and AR. A $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Of the 156 cancer spots investigated, 122 had a primary Gleason grade of <4 and the remaining 34 were ≥4. Akt expression was localized mainly in the cytoplasm (Fig. 1A). In rare cases, Akt was localized in the nuclei. The expression of p-Akt was similar (Fig. 1C). AR expression was located in both the cytoplasm and nuclei (Fig. 1E). Ki67 expression was localized in the nuclei (Fig. 1G). The median (range; interquartile range, IQR) Akt and p-Akt staining scores were 120 (60–300; IQR 100–200) and 100 (0–300; IQR 100–200), respectively. There was a significant positive correlation between p-Akt and Akt expression levels (Spearman's correlation, $r = 0.644$, $P < 0.001$; Fig. 2). The median (range) AR-staining score was 1 (1–3; IQR 1–2) and Ki67 LI was 2.85 (0–49.7; IQR 1.15–8.00).

The staining intensity of Akt, p-Akt, AR, and Ki67 in each cancer spot was classified as low or high. In tumours with a primary Gleason grade of <4, 58.2% of spots had a low staining score for Akt and 62.3% had a low staining score for p-Akt. By contrast, in tumours with a primary Gleason grade of ≥4, 73.5% of spots had a high staining score for Akt and 58.8% had a high staining score for p-Akt. There was a highly significant difference in the staining scores for Akt and p-Akt between tumours with a primary Gleason grade of <4 and tumours with a primary Gleason grade ≥4 (Fisher's exact test, $P = 0.002$ and $P = 0.032$, respectively; Table 2).

In all, 79 spots (50.6%) had low AR expression and the remaining 77 (49.4%) had high AR expression. In the spots with low AR expression, 75.9% had low Akt expression and 74.7% had low p-Akt expression. In the spots with high AR expression, 74.0% had high Akt expression and 59.7% had high p-Akt expression. There was a highly significant difference in the staining score for Akt and p-Akt between tumours with low AR expression and tumours with high AR expression (both $P < 0.001$, Table 2).There was also a strong relationship between the Ki67 LI and the expression of Akt and p-Akt ($P < 0.001$ and $P = 0.001$, respectively; Table 2).

The 5-year PSA-defined disease-free survival rate for the 52 patients was 59.6% (31

© 2007 THE AUTHORS
JOURNAL COMPILATION © 2007 BJU INTERNATIONAL

TABLE 2 *Expression of Akt and p-Akt in relation to primary Gleason grade, AR expression and Ki-67 LI in each cancer spot (156 spots)*

Variable, n (%)	Akt expression (score)			p-Akt expression (score)		
	low intensity*	high intensity†	P‡	low intensity*	high intensity†	P‡
Primary Gleason grade						
low grade (<4)	71 (58.2)	51 (41.8)	0.002	76 (62.3)	46 (37.7)	0.032
high grade (≥4)	9 (26.5)	25 (73.5)		14 (41.2)	20 (58.8)	
AR expression (score)						
low intensity (<1.6)	60 (75.9)	19 (24.1)	<0.001	59 (74.7)	20 (25.3)	<0.001
high intensity (≥1.6)	20 (26.0)	57 (74.0)		31 (40.3)	46 (59.7)	
Ki-67 LI (%)						
low intensity (<5)	69 (71.1)	28 (28.9)	<0.001	64 (66.0)	33 (34.0)	0.008
high intensity (≥5)	11 (18.6)	48 (81.4)		26 (44.1)	33 (55.9)	

**low intensity score <140; †high intensity score ≥ 140; ‡Fisher's exact test.*

patients). Univariate analysis showed that Akt, p-Akt, AR expression, Ki67 LI, pT stage, primary Gleason score, surgical margin, TMA Gleason sum and TMA primary Gleason score were significant factors for PSA-defined disease-free survival (Table 3). The relative importance of each variable was determined by multivariate Cox proportional-hazards model analysis. Stepwise inclusion of variables in the model showed that the significant factors were primary Gleason score ($P = 0.043$, hazard ratio, HR, 2.59), surgical margin status ($P = 0.006$, HR 8.39) and Ki67 LI ($P < 0.001$, HR 6.58) (Table 3).

TABLE 3 *Univariate and multivariate analysis (Cox regression models) for PSA-defined treatment failure*

Variable	HR (95% CI)	P
Univariate analysis		
Age (<70 vs ≥70 years)	0.70 (0.29, 1.66)	0.414
PSA level (≥10 vs <10 ng/ml)	2.07 (0.86, 4.99)	0.107
pT stage (≥T3 vs <T3)	4.61 (1.07, 20)	0.040*
Gleason sum (≥7 vs <7)	1.43 (0.9, 2.28)	0.130
Primary Gleason score (≥4 vs <4)	3.26 (1.37, 7.8)	0.008*
TMA Gleason sum (≥7 vs <7)	3.67 (1.08, 12.49)	0.037*
TMA primary Gleason score (≥4 vs <4)	6.17 (2.07, 18.45)	0.011*
Surgical margin (positive vs negative)	5.79 (1.34, 24.93)	0.018*
Akt score (≥140 vs <140)	2.53 (1.02, 6.29)	0.046*
p-Akt score (≥140 vs <140)	2.54 (1.02, 6.31)	0.044*
AR (high vs low)	2.45 (0.99, 6.11)	0.049*
Ki67 LI (positive vs negative)	6.64 (2.40, 18.33)	<0.001*
Multivariate analysis		
Primary Gleason score (≥4 vs <4)	2.59 (1.03, 6.53)	0.043*
Surgical margin (positive vs negative)	8.39 (1.86, 37.86)	0.006*
Ki67 LI (positive vs negative)	6.58 (2.26, 19.15)	<0.001*

**P < 0.05.*

DISCUSSION

The present results show the importance of Akt expression and activation as molecular markers for malignant biological features of prostate cancer in the Japanese population. The expression and activation of Akt was increased in high Gleason grade prostate cancers. Liao *et al.* [14] also reported that an increase of Akt expression correlated with Gleason pattern in human prostate cancer, while Le Page *et al.* [18] reported that there was no correlation between Akt-1, Akt-2, or Akt-3 and Gleason score, surgical grade or pathological stage. Expression of p-Akt was reported by Malik *et al.* [15] to be significantly greater in Gleason grades 8–10 vs all other grades, while Ayala *et al.* [17] showed that p-Akt expression was correlated only with clinical staging of tumour, and Le Page *et al.* [18] showed that p-Akt overexpression was correlated only with preoperative PSA level. The reasons for the discrepancies among these results are unknown, but differences in the patient cohorts or in statistical analysis might be contributory.

Fenic *et al.* [22] reported that when comparing prostate intraepithelial neoplasia, primary carcinomas and metastases as ordered groups (with increasing malignancy), both PTEN mRNA ($r = -0.49$, $P = 0.06$) and its protein ($r = -0.36$, $P = 0.09$) showed a progressive decreasing trend. PTEN protein expression was moderate to strong in 27 of 58 primary tumours (46.5%), weak in 24 (41.3%), and negative in eight (13.8%). Of 15 metastases, PTEN protein expression was moderate to strong in four, weak in six, and negative in five [22]. In the present early-stage prostate cancer tissues, ≈49% of cancer spots showed high expression of Akt and there was a significant correlation between the expression of Akt and p-Akt ($r = 0.644$, $P < 0.001$). These results suggest that both the loss of PTEN and the increase in Akt expression might activate Akt in early-stage human prostate cancer and play an important role in the poor differentiation of prostate cancer cells. However, the precise role of this early up-regulation of Akt needs further examination.

© 2007 THE AUTHORS
JOURNAL COMPILATION © 2007 BJU INTERNATIONAL

PROSTATE CANCER IN JAPANESE MEN

In the present study, expression of Akt and p-Akt were strongly related with the Ki67 LI, the cell proliferation antigen. Akt is a well-known regulator of cell survival, promoting cell survival by phosphorylating and inactivating the pro-apoptotic protein BAD [23]. Akt also stimulates cell cycle progression by suppressing AFX-mediated transcription of target genes such as the cyclin-dependent kinase inhibitor $p27^{KIP1}$ [24]. Thus, activation of Akt regulates not only survival but also proliferation of cancer cells. Ghosh *et al.* [25] verified these results *in vitro* using LNCaP cells and its androgen-independent clone C4-2, and *in vivo* using human prostate cancer tissues. C4-2 cells expressed higher Akt activation and increased proliferation than LNCaP cells, similar to cases with poor clinical outcome [25]. They also reported in an immunohistochemical study of 74 human prostate cancer specimens that there was a strong correlation between the expression of p-Akt and the Ki67 LI [25], which is compatible with the present results.

In the present immunohistochemical study, there was a strong relationship between the expression of Akt or p-Akt and AR expression. Manin *et al.* [13] showed that AR protein levels are greatly reduced after treatment of LNCaP cells with the PI3K/Akt pathway inhibitor LY294002. LY294002 does not alter AR mRNA accumulation, suggesting that translational or post-translational mechanisms might be the main factors responsible for decreased AR expression. Consistent with this possibility, it was reported that Akt activates mRNA translation by phosphorylation and inactivation of the translational repressor eIF4E-binding proteins [26]. The mammalian target of rapamycin, which is important in the regulation of protein translation, is a direct target for Akt [27] and might be the mediator of the growth factor's effects on AR protein expression. Recently Xin *et al.* [28] suggested synergy of Akt and AR exists in the progression of prostate cancer. In the present study, there was a significant relationship between Akt/p-Akt expression and AR expression, suggesting that the PI3K/Akt pathway might control AR-mediated progression of prostate cancer. To our knowledge, this is the first immunohistochemical study showing a strong relationship between the expression of Akt or p-Akt and the expression of AR in prostate cancer. Previously published data show that the significance of the AR in prostate cancer after RP remains limited and inconclusive. Li *et al.* [29] reported that patients with higher levels of AR that had a RP had a higher degree of malignancy, more advanced disease progression, and worse biological recurrence-free survival, while another study showed that low AR expression correlated with histopathological grade and indicated a poor prognosis [30]. Although the correlations between the expression of AR and prognosis are not fully understood, considering that the androgen/AR complexes are known to promote tumour cell proliferation and survival [31], it is reasonable to think that hormonally naïve prostate cancer treated with RP might take advantage of higher AR status, which eventually might lead to enhanced AR activity, resulting in more growth advantage under a low androgen environment due to ageing [32]. Moreover, this aggressiveness was confirmed by a significant positive relationship between AR immunostaining and Ki67 LI in our previous study [21] and a study by Li *et al.* [29].

Kreisberg *et al.* [16] reported that in a group with weak p-Akt staining (score 1–100) the PSA failure rate was half, whereas, in a moderate staining group (101–200) the rate was 66.7%, and in a strong staining group (201–300) it was 91.3%. Increased p-Akt was an independent predictor of the probability of PSA failure. Although p-Akt was not an independent prognostic factor according to multivariate analysis in the present study, a similar tendency was observed: in the weak p-Akt staining group the PSA failure rate was 23.5%, whereas in the moderate and strong staining groups the rates were 43.3 and 60.0%, respectively.

In conclusion, immunohistochemical analysis of prostate cancer specimens from Japanese men showed that the aggressive form of prostate cancer is accompanied by increased levels of Akt and p-Akt. The close correlation between the expression of Akt and p-Akt shows that the higher expression of Akt might increase its activity and play an important role in the poor differentiation of prostate cancer cells. Although Akt and p-Akt were not independent prognostic factors according to our multivariate analysis, the expression of Akt and p-Akt were related with tumour grade, AR-staining score and the Ki67 LI, and are therefore thought to be molecular markers for malignant biological features of prostate cancer. We think this is the first report from an Asian country showing a strong relationship between the aggressiveness of prostate cancer and expression of Akt and p-Akt. Despite the previously noted differences in gene alterations between prostate cancers from Japan and Western countries, the present study highlights the similarities of Akt and p-Akt alterations between these populations. One potential implication of the present study is that Akt and AR alterations are more central in prostate-cancer biology, in comparison to the other gene alterations, which might be linked to environmental factors. Further investigation of the Akt pathway might therefore reveal its involvement in the progression of prostate cancer.

CONFLICT OF INTEREST

None declared.

REFERENCES

1 **Landis SH, Murray T, Bolden S, Wingo PA.** Cancer statistics, 1999. *CA Cancer J Clin* 1999; **49**: 8–31
2 **Catalona WJ.** Management of cancer of the prostate. *N Engl J Med* 1994; **331**: 996–1004
3 **Isaacs JT.** Apoptosis: translating theory to therapy for prostate cancer. *J Natl Cancer Inst* 2000; **92**: 1367–9
4 **Li J, Yen C, Liaw D *et al.*** PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 1997; **275**: 1943–7
5 **Steck PA, Pershouse MA, Jasser SA *et al.*** Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* 1997; **15**: 356–62
6 **Stambolic V, Suzuki A, de la Pompa JL *et al.*** Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 1998; **95**: 29–39
7 **Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ.** Akt-1/PKBα is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J Biol Chem* 2001; **276**: 38349–52
8 **Cho H, Mu J, Kim JK *et al.*** Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt-2 (PKBβ). *Science* 2001; **292**: 1728–31

© 2007 THE AUTHORS
JOURNAL COMPILATION © 2007 BJU INTERNATIONAL

SHIMIZU CTA

9 **Easton RM, Cho H, Roovers K *et al.*** Role for Akt-3/protein kinase Bγ in attainment of normal brain size. *Mol Cell Biol* 2005; **25**: 1869–78

10 **Majumder PK, Yeh JJ, George DJ *et al.*** Prostate intraepithelial neoplasia induced by prostate restricted Akt activation: the MPAKT model. *Proc Natl Acad Sci USA* 2003; **100**: 7841–6

11 **Lin J, Adam RM, Santiestevan E, Freeman MR.** The phosphatidylinositol 3′-kinase pathway is a dominant growth factor-activated cell survival pathway in LNCaP human prostate carcinoma cells. *Cancer Res* 1999; **59**: 2891–7

12 **Graff JR, Konicek BW, McNulty AM *et al.*** Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing $p27^{Kip1}$ expression. *J Biol Chem* 2000; **275**: 24500–5

13 **Manin M, Baron S, Goossens K *et al.*** Androgen receptor expression is regulated by the phosphoinositide 3-kinase/Akt pathway in normal and tumoral epithelial cells. *Biochem J* 2002; **366**: 729–36

14 **Liao Y, Grobholz R, Abel U *et al.*** Increase of AKT/PKB expression correlates with Gleason pattern in human prostate cancer. *Int J Cancer* 2003; **107**: 676–80

15 **Malik SN, Brattain M, Ghosh PM *et al.*** Immunohistochemical demonstration of phospho-Akt in high Gleason grade prostate cancer. *Clin Cancer Res* 2002; **8**: 1168–71

16 **Kreisberg JI, Malik SN, Prihoda TJ *et al.*** Phosphorylation of Akt (Ser^{473}) is an excellent predictor of poor clinical outcome in prostate cancer. *Cancer Res* 2004; **64**: 5232–6

17 **Ayala G, Thompson T, Yang G *et al.*** High levels of phosphorylated form of Akt-1 in prostate cancer and non-neoplastic prostate tissues are strong predictors of biochemical recurrence. *Clin Cancer Res* 2004; **10**: 6572–8

18 **Le Page C, Koumakpayi IH, Alam-Fahmy M, Mes-Masson AM, Saad F.** Expression and localisation of Akt-1, Akt-2 and Akt-3 correlate with clinical outcome of prostate cancer patients. *Br J Cancer* 2006; **94**: 1906–12

19 **Shiraishi T, Muneyuki T, Fukutome K *et al.*** Mutations of ras genes are relatively frequent in Japanese prostate cancers: pointing to genetic differences between populations. *Anticancer Res* 1998; **18**: 2789–92

20 **Sim HG, Cheng CW.** Changing demography of prostate cancer in Asia. *Eur J Cancer* 2005; **41**: 834–45

21 **Inoue T, Segawa T, Shiraishi T *et al.*** Androgen receptor, Ki67, and p53 expression in radical prostatectomy specimens predict treatment failure in Japanese population. *Urology* 2005; **66**: 332–7

22 **Fenic I, Franke F, Failng K, Steger K, Woenckhaus J.** Expression of PTEN in malignant and non-malignant human prostate tissues: comparison with p27 protein expression. *J Pathol* 2004; **203**: 559–66

23 **Kandel ES, Hay N.** The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp Cell Res* 1999; **253**: 210–29

24 **Medema RH, Kops GJ, Bos JL, Burgering BM.** AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through $p27^{kip1}$. *Nature* 2000; **404**: 782–7

25 **Ghosh PM, Malik SN, Bedolla RG *et al.*** Signal transduction pathways in androgen-dependent and -independent prostate cancer cell proliferation. *Endocr Relat Cancer* 2005; **12**: 119–34

26 **Gingras AC, Kennedy SG, O'Leary MA, Sonenberg N, Hay N.** 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. *Genes Dev* 1998; **12**: 502–13

27 **Nave BT, Ouwens M, Withers DJ, Alessi DR, Shepherd PR.** Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J* 1999; **344**: 427–31

28 **Xin L, Teitell MA, Lawson DA, Kwon A, Mellinghoff IK, Witte ON.** Progression of prostate cancer by synergy of AKT with genotropic and nongenotropic actions of the androgen receptor. *Proc Natl Acad Sci USA* 2006; **103**: 7789–94

29 **Li R, Wheeler T, Dai H, Frolov A, Thompson T, Ayala G.** High level of androgen receptor is associated with aggressive clinicopathologic features and decreased biochemical recurrence-free survival in prostate cancer patients treated with radical prostatectomy. *Am J Surg Pathol* 2004; **28**: 928–34

30 **Schafer W, Funke PJ, Kunde D, Rausch U, Wennemuth G, Stutzer H.** Intensity of androgen and epidermal growth factor receptor immunoreactivity in sample of radical prostatectomy as prognostic indicator: correlation with clinical data of long-term observations. *J Urol* 2006; **176**: 532–7

31 **Isaacs JT.** Antagonistic effect of androgen on prostatic cell death. *Prostate* 1984; **5**: 545–57

32 **Iwamoto T, Yanase T, Koh E *et al.*** [Reference ranges of serum total and free testosterone in Japanese male adults]. *Nippon Hinyokika Gakkai Zasshi* 2004; **95**: 751–60

Correspondence: Osamu Ogawa, Department of Urology, Kyoto University Graduate School of Medicine, 54 Kawaracho Shogoin Sakyoku, Kyoto, 606-8507, Japan.
e-mail: ogawao@kuhp.kyoto-u.ac.jp

Abbreviations: **AAT**, androgen-ablation therapy; **PI3K**, phosphatidylinositol 3-kinase; **AR**, androgen receptor; **p-Akt**, phosphorylated Akt; **LI**, labelling index; **RP**, radical prostatectomy; **TMA**, tissue microarray; **IQR**, interquartile range; **HR**, hazard ratio.

© 2007 THE AUTHORS
JOURNAL COMPILATION © 2007 BJU INTERNATIONAL

international journal of andrology ISSN 0105-6263

ORIGINAL ARTICLE

Testosterone decreased urinary-frequency in nNOS-deficient mice

Satoru Muto, Mitsuko Yasuda, Yutaka Kamiyama, Hisamitsu Ide and Shigeo Horie

Department of Urology, Teikyo University, Itabashi-Ku, Tokyo, Japan

Keywords:
bladder overactivity, neuronal nitric oxide synthase, neuronal nitric oxide synthase null mutant mice, testosterone replacement therapy, urinary frequency

Correspondence:
Shigeo Horie, MD, Department of Urology, Teikyo University, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan.
E-mail: shorie@med.teikyo-u.ac.jp

Received 13 December 2006; revised 30 January 2007; accepted 5 February 2007

doi:10.1111/j.1365-2605.2007.00763.x

Summary

To observe the effect of testosterone on the frequency of urination in mice lacking neuronal nitric oxide synthase ($nNOS^{-/-}$), we compared the urination patterns between unanaesthetized male wild-type ($n = 27$) and $nNOS^{-/-}$ mice ($n = 50$) with or without testosterone treatment. Compared with wild-type mice, $nNOS^{-/-}$ mice showed a greater frequency of urination during a 24-h observation period (3.0 vs. 5.4 times/day, $p < 0.0001$) without any significant difference in the total voided volume or the functional voiding capacity. While testosterone treatment did not affect the urination patterns in wild-type, it decreased the daytime frequency of urination (5.4 vs. 3.7 times, $p = 0.0198$) and the nighttime urination (4.4 vs. 2.9 times, $p = 0.039$) in $nNOS^{-/-}$ mice. The $nNOS^{-/-}$ mice can be a useful animal model for urinary frequency. Testosterone improved the functional abnormalities in the voiding of $nNOS^{-/-}$ mice.

Introduction

The relaxation of bladder outlet regions on voiding and the maintenance of low pressure during urinary storage are prerequisite conditions for normal micturition. An accumulating body of evidence indicates that nitric oxide (NO) is an important physiological inhibitory neurotransmitter that mediates relaxation in the smooth muscle component (Andersson *et al.*, 1992; Thornbury *et al.*, 1992; Persson & Andersson, 1994). Nitric oxide is produced as a result of the conversion of the substrate L-arginine to L-citrulline by three isoforms of the enzyme NO synthase (NOS). The mechanism underlying NO-induced smooth muscle relaxation was found to be calcium-dependent, and cyclic guanosine monophosphate (cGMP) was identified as the second messenger. The individual NOS isoforms (eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase and iNOS, inducible nitric oxide synthase), have a unique subcellular location, structure, kinetics, regulation and function. There is increasing evidence that alterations in the NO–cGMP pathway play an important role in the development of lower urinary tract syndrome (LUTS) (Andersson *et al.*, 2002; Mamas *et al.*, 2003; Chertin *et al.*, 2004; Hedlund, 2005). Neuronally released NO has been suggested to be involved in the relaxation of the urethral smooth muscle during micturition (Buga *et al.*, 1989; Andersson & Persson, 1995; Burnett, 1995). By inhibiting the production of NO, both bladder hyperactivity and a decreased bladder capacity have been demonstrated (Persson *et al.*, 1992).

Indeed a targeted disruption of the nNOS gene has been demonstrated to result in voiding abnormalities (Burnett *et al.*, 1997). In $nNOS^{-/-}$ mice, urinary bladders develop hypertrophy because of deficient outflow relaxation. The urinary bladder and urethra do not relax in response to electrical field stimulation or L-arginine, the amino-acid substrate for NO. The $nNOS^{-/-}$ mice have urinary frequency and a decreased threshold of afferent firing of the bladder detrusor, thus indicating that nNOS is responsible for the relaxation of the bladder outlet regions on voiding and the maintenance of low pressure in urinary storage. These features observed in the abnormal urination of $nNOS^{-/-}$ mice are thus considered to be a plausible model for human overactive bladder.

Recently, several studies have shown testosterone (T) to induce the relaxation of smooth muscle cells by modulating both the nNOS activity and cGMP level. Androgens play a pivotal role in the erectile function by

© 2007 The Authors
Journal compilation © 2007 Blackwell Publishing Ltd

regulating both cGMP formation by NOS and degradation by phosphodiesterase 5 (PDE5) (Morelli *et al.*, 2005; Vignozzi *et al.*, 2005). In addition, testosterone has direct vasoactive properties. Testosterone has been shown to cause a dose dependant effect on the relaxation of vascular smooth muscles (Webb *et al.*, 1990; English *et al.*, 2001). It is caused either by a direct effect on the vascular smooth muscle, or by an effect on potassium channel (Deenadayalu *et al.*, 2001; English *et al.*, 2002). These lines of evidence prompted us to investigate the role of testosterone in the relaxation of bladder smooth muscle. To evade the effect of nNOS in the relaxation of bladder, $nNOS^{-/-}$ mice was examined to see the role of T. The purpose of the current study was to examine the effect of testosterone on the abnormal voiding of $nNOS^{-/-}$ mice.

Materials and methods

Animals

Experiments were performed in nNOS null mutant mice ($nNOS^{-/-}$) generated by Huang *et al.* (1993) and their wild-type littermates. All wild-type littermates ($n = 27$) and homozygous $nNOS^{-/-}$ males ($n = 50$) of C57Bl/6 strains with targeted disruption of exon2 of the nNOS gene, used in this study were derived from a heterozygous breeding pair. Both groups were housed and studied under identical conditions. At weaning, the animals were ear-tagged. Genomic DNA was extracted from the tails after digestion with proteinase K and E, and purification of DNA with ethanol. The genotype of each DNA sample was then determined by testing for the presence of wild-type or modified nNOS sequences by use of PCR. Animals were maintained on standard rodent chow and tap water at 23 ± 1 °C with a 12/12-h light-dark cycle (lights on at 6:00 AM). The mice always had free access to food and tap water. All care and handling of the animals were carried out in accordance with institutional guidelines.

Voiding behaviour

Male $nNOS^{-/-}$ and wild-type littermates aged 8–13 months and weighing between 30 and 40 gm were used in this study. Voiding studies were carried out after a 3-day period of acclimatization to the laboratory. Individual mice were placed in a mouse micturition cage. The urine collection funnel and faecal separation screen were placed on the bottom of the cage. Each scale was monitored using a remote computer running an application that permits recording information sent by the scale to a resolution of 1 sec. The voiding volume is expressed as weight with a resolution of 10 mg. When assuming a urine specific gravity of approximately, 1.1 mg urine has a volume of 1 mL.

This assumption was reasonable because animals with polydipsia tend to produce extremely dilute urine. The total amount of urine voided in 1 day was accumulated into a container. Voiding was studied continuously for a 10-day period.

Testosterone administration

After stable baseline voiding patterns had been established, 3.6 mg/g body weight testosterone enanthate (Teikoku Hormone Mfg, Tokyo, Japan) diluted in sesame oil or other vehicles were singly administered intraperitoneally to male wild-type littermates and $nNOS^{-/-}$. This dose was selected based on previous studies that examined the effect of androgen on the gene expression in the prostate (Mirosevich *et al.*, 1999, 2001). The voiding pattern of the treated mice was examined 1-week later. We examined the urinary frequency, functional bladder capacity and the urinary volume.

Statistics

The results are generally reported as the mean ± SD. Student's unpaired two-tailed t test was used compared with the micturition parameters between the control and obstructed groups, as well as between the wild-type littermates and $nNOS^{-/-}$. One-way ANOVA was used to analyse to compare the differences of the parameters among four groups (wild-type with or without testosterone and $nNOS^{-/-}$ with or without T). A probability level of <0.05 was considered to be significant.

Results

The micturitional characteristics of wild-type and $nNOS^{-/-}$ mice

The amount of urine output per day (0.84 ± 0.40 mL vs. 1.09 ± 0.55 mL, $p = 0.282$) and the functional bladder capacity (defined as the voided volume per void) (0.28 ± 0.17 mL vs. 0.21 ± 0.12 mL, $p = 0.061$) did not differ substantially between the wild-type and $nNOS^{-/-}$ mice. Within comparison to the wild-type mice, the $nNOS^{-/-}$ mice showed a greater frequency of urination during the 24-h observation period (3.00 ± 1.00 times vs. 5.40 ± 0.97 times, $p < 0.0001$) and also during the nocturnal period (2.50 ± 0.85 times vs. 4.40 ± 1.27 times, $p = 0.001$) (Table 1).

However, no significant differences were seen in the frequency of daytime voiding between the wild type and $nNOS^{-/-}$ mice (0.50 ± 0.71 times vs. 0.60 ± 0.70 times, $p = 0.750$).

© 2007 The Authors
Journal compilation © 2007 Blackwell Publishing Ltd

Table 1 The number of voids in wild-type and nNOS$^{-/-}$ mice

	WT (n = 27)	KO (n = 50)	p-value
Capacity			
Total micturition volume (mL/24 h)	0.84 ± 0.40	1.09 ± 0.55	0.282
Functional capacity (mL/void)	0.28 ± 0.17	0.21 ± 0.12	0.061
	WT (n = 10)	KO (n = 10)	
frequency			
No. of voids/24 h	3.00 ± 1.00	5.40 ± 0.97	<0.0001
No. of daytime voids	0.50 ± 0.71	0.60 ± 0.70	0.750
No. of nightime voids	2.50 ± 0.85	4.40 ± 1.27	0.001

WT, wild-type; KO, neuronal nitric oxide synthase (nNOS$^{-/-}$).

Effects of testosterone on the urinary frequency of nNOS$^{-/-}$ and the wild-type mice

Testosterone treatment did not affect either the amount of urine output per day (wild-type; 0.84 ± 0.40 mL vs. 0.64 ± 0.57 mL, p = 0.420, nNOS$^{-/-}$; 1.09 ± 0.55 mL vs. 1.01 ± 0.59 mL, p = 0.782) or the functional bladder capacity (wild-type; 0.28 ± 0.17 mL vs. 0.24 ± 0.13 mL, p = 0.331, nNOS$^{-/-}$; 0.21 ± 0.12 mL vs. 0.27 ± 0.10 mL, p = 0.057) in the wild-type and nNOS$^{-/-}$ mice. Regarding the frequency of daytime voiding, there were no significant differences before and after testosterone administration in the wild-type and nNOS$^{-/-}$ mice (wild-type; 0.50 ± 0.71 times vs. 1.33 ± 1.37 times, p = 0.127, nNOS$^{-/-}$; 0.60 ± 0.70 times vs. 0.86 ± 0.69 times, p = 0.465). However, in the nNOS$^{-/-}$ mice, testosterone administration significantly decreased the frequency of urination during both the 24-h observation period (5.40 ± 0.97 times vs. 3.71 ± 1.70 times, p = 0.020) and the nocturnal period (4.40 ± 1.27 times vs. 2.86 ± 1.86 times, p = 0.039). By one-way ANOVA, there were statistical differences in both the 24-h observation period (p =0.018) and the nocturnal period (p < 0.001) among four groups (wild-type with or without testosterone nNOS$^{-/-}$ mice with or without T) (Table 2).

Discussion with conclusions

In the present study, we confirmed the previous study that nNOS$^{-/-}$ mice had urinary frequency with a decreased functional urinary bladder capacity. Thus nNOS$^{-/-}$ mice can be a useful animal model for the exploration of the treatment of LUTS. Several lines of evidence may allow the speculation for the underlying mechanisms of the effect of the testosterone treatment on the voiding abnormalities of nNOS$^{-/-}$ mice.

First, testosterone has been shown to cause a dose-dependant effect on the relaxation of vascular smooth muscles (Webb *et al.*, 1990; English *et al.*, 2001). However, it remained unknown whether testosterone relaxes smooth muscle cell in a tonic manner. Secondly, there is evidence that androgens can regulate the expression of NOS enzymes in the corporeal tissue (Chamness *et al.*, 1995; Park *et al.*, 1999). As a result, in nNOS$^{-/-}$ mice, testosterone might possibly augment the expression of eNOS to compensate for the loss of nNOS. A previous study demonstrated that androgens maintain the erectile response by the pathways that are independent of NO but involve the synthesis of cGMP (Reilly *et al.*, 1997). Those hypotheses warrant further studies. The association of the late-onset hypogonadism and urinary frequency has been reported in literature. The findings of this study may indicate that testosterone potentially has a therapeutic effect on an overactive bladder by decreasing the nNOS expression in ageing males. The nNOS$^{-/-}$ mice showed an increased urinary frequency. Testosterone treatment significantly improved the urinary frequency for nNOS$^{-/-}$ mice. These findings may indicate that testosterone can improve bladder overactivity because of the loss of nNOS. Androgen replacement may therefore be a potentially useful and novel pharmacological target for patients with a decreased level of NO thus leading to urinary frequency.

Table 2 The voiding function in wild-type and nNOS$^{-/-}$ mice before and after testosterone administration

	WT (n = 27)	WT + T (n = 19)	p-value	KO (n = 50)	KO + T (n = 26)	p-value
Capacity						
Total micturition volume (mL/24 h)	0.84 ± 0.40	0.64 ± 0.57	0.420	1.09 ± 0.55	1.01 ± 0.59	0.782
Functional capacity (mL/void)	0.28 ± 0.17	0.24 ± 0.13	0.331	0.21 ± 0.12	0.27 ± 0.10	0.057
	WT (n = 10)	WT + T (n = 7)		KO (n = 10)	KO + T (n = 7)	
Frequency						
No. of voids/24 h	3.00 ± 1.00	2.71 ± 2.22	0.734	5.40 ± 0.97	3.71 ± 1.70	0.020
No. of daytime voids	0.50 ± 0.71	1.33 ± 1.37	0.127	0.60 ± 0.70	0.86 ± 0.69	0.465
No. of nightime voids	2.50 ± 0.85	1.33 ± 1.21	0.059	4.40 ± 1.27	2.86 ± 1.86	0.039

WT, wild-type; WT + T, wild-type after testosterone administration; KO, neuronal nitric oxide synthase (nNOS$^{-/-}$); KO + T, nNOS$^{-/-}$ after testosterone administration.

© 2007 The Authors
Journal compilation © 2007 Blackwell Publishing Ltd

References

Andersson, K. E. & Persson, K. (1995) Nitric oxide synthase and the lower urinary tract: possible implications for physiology and pathophysiology. *Scandinavian Journal of Urology and Nephrology* 175, 43–53.

Andersson, K. E., Garcia Pascual, A., Persson, K., Forman, A. & Tottrup, A. (1992) Electrically-induced, nerve-mediated relaxation of rabbit urethra involves nitric oxide. *Journal of Urology* 147, 253–259.

Andersson, K. E., Chapple, C. R. & Hofner, K. (2002) Future drugs for the treatment of benign prostatic hyperplasia. *World Journal of Urology* 19, 436–442.

Buga, G. M., Gold, M. E., Wood, K. S., Chaudhuri, G. & Ignarro, L. J. (1989) Endothelium-derived nitric oxide relaxes non-vascular smooth muscle. *European Journal Pharmacology* 161, 61–72.

Burnett, A. L. (1995) Nitric oxide control of lower genitourinary tract functions: s review. *Urology* 45, 1071–1083.

Burnett, A. L., Calvin, D. C., Chamness, S. L., Liu, J. X., Nelson, R. J., Klein, S. L., Dawson, V. L., Dawson, T. M. & Snyder, S. H. (1997) Urinary bladder-urethral sphincter dysfunction in mice with targeted disruption of nNOS models idiopathic voiding disorders in humans. *Nature Medicine* 3, 571–574.

Chamness, S. L., Ricker, D. D., Crone, J. K., Dembeck, C. L., Maguire, M. P., Burnett, A. L. & Chang, T. S. (1995) The effect of androgen on NOS in the male reproductive tract of the rat. *Fertility and Sterility* 63, 1101–1107.

Chertin, B., Rolle, U., Solari, V., Cascio, S. & Puri, P. (2004) The role of nitric oxide in bladder urothelial injury after bladder outlet obstruction. *BJU International* 94, 392–399.

Deenadayalu, V. P., White, R. E., Stallone, J. N., Gao, X. & Garcia, A. J. (2001) Testosterone relaxes coronary arteries by opening the large-conductance, calcium-activated potassium channel. *American Journal of Physiology Heart and Circulatory Physiology* 281, H1720–1727.

English, K. M., Jones, R. D., Jones, T. H., Morice, A. H. & Channer, K. S. (2001) Gender differences in the vasomotor effects of different steroid hormones in rat pulmonary and coronary arteries. *Hormone and Metabolic Research* 33, 645–652.

English, K. M., Jones, R. D., Jones, T. H., Morice, A. H. & Channer, K. S. (2002) Testosterone acts as a coronary vasodilator by a calcium channel antagonist action. *Journal of Endocrinological Investigation* 25, 455–458.

Hedlund, P. (2005) Nitric oxide/cGMP-mediated effects in the outflow region of the lower urinary tract: is there a basis for pharmacological targeting of cGMP? *World Journal of Urology* 23, 362–367.

Huang, P. L., Dawson, T. M., Bredt, D. S., Snyder, S. H. & Fishman, M. C. (1993) Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* 75, 1273–1286.

Mamas, M. A., Reynard, J. M. & Brading, A. F. (2003) Nitric oxide and the lower urinary tract: current concepts, future prospects. *Urology* 61, 1079–1085.

Mirosevich, J., Bentel, J. M., Zeps, N., Redmond, S. L., D'Antuono, M. F. & Dawkins, H. J. (1999) Androgen receptor expression of proliferating basal and luminal cells in adult murine ventral prostate. *The Journal of Endocrinology* 162, 341–350.

Mirosevich, J., Bentel, J. M. & Dawkins, J. S. (2001) Regulation of caltrin mRNA expression by androgens in the murine prostate. *Journal of Andrology* 22, 449–457.

Morelli, A., Filippi, S., Zhang X. H., Luconi, M., Vignozzi, L., Mancina, R. & Maggi, M. (2005) Hormonal regulation of peripheral regulatory mechanisms in erection. *International Journal of Andrology* 28, 23–27.

Park, K. H., Kim, S. W., Kim, K. D. & Paick, J. S. (1999) Effects of androgens on the expressions of nitric oxide synthase mRNAs in rat corpus cavernosum. *BJU International* 83, 327–333.

Persson, K. & Andersson, K. E. (1994) Non-adrenergic, non-cholinergic relaxation and levels of cyclic nucleotides in rabbit lower urinary tract. *European Journal of Pharmacology* 268, 159–167.

Persson, K., Igawa, Y., Mattiasson, A. & Andersson, K. E. (1992) Effects of inhibition of the L-arginine/nitric oxide pathway in urinary tract in vivo and in vitro. *British Journal of Pharmacology* 107, 178–184.

Reilly, C. M., Lewis, R. W., Stopper, V. S. & Mills, T. M. (1997) Androgenic maintenance of the rat erectile response via a non-nitric oxide-dependent pathway. *Journal of Andrology* 18, 588–594.

Thornbury, K. D., Hollywood, M. A. & McHale, N. G. (1992) Mediation by nitric oxide of neurogenic relaxation of the urinary bladder neck muscle in sheep. *The Journal of Physiology* 451, 133–144.

Vignozzi, L., Corona, G., Petrone, L., Filippi, S., Morelli, A. M., Forti, G. & Maqqi, M. (2005) Testosterone and sexual activity. *Journal of Endocrinological Investigation* 28, 39–44.

Webb, C. M., McNeill, J. G., Hayward, C. S., de Zeigler, D. & Collins, P. (1990) Effects of testosterone on coronary vasomotor regulation in men with coronary heart disease. *Circulation* 100, 1690–1696.

© 2007 The Authors
Journal compilation © 2007 Blackwell Publishing Ltd

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications xxx (2007) xxx–xxx
BBRC
www.elsevier.com/locate/ybbrc

Tyk2 expression and its signaling enhances the invasiveness of prostate cancer cells

Hisamitsu Ide [a], Takashi Nakagawa [a], Yuichi Terado [b], Yutaka Kamiyama [a], Satoru Muto [a], Shigeo Horie [a,*]

[a] *Department of Urology, Teikyo University School of Medicine, 2-11-1 Kaga, 2chome, Itabashi-ku, Tokyo 173-8605, Japan*
[b] *Department of Pathology, Kyorin University School of Medicine, Mitaka-city, Tokyo, Japan*

Received 13 August 2007

Abstract

Protein tyrosine kinase plays a central role in the proliferation and differentiation of various types of cells. One of these protein kinases, Tyk2, a member of the Jak family kinases, is known to play important roles in receptor signal transduction by interferons, interleukins, growth factors, and other hormones. In the present study, we investigated Tyk2 expression and its role in the growth and invasiveness of human prostate cancer cells. We used a small interfering RNA targeting Tyk2 and an inhibitor of Tyk2, tyrphostin A1, to suppress the expression and signaling of Tyk2 in prostate cancer cells. We detected mRNAs for Jak family kinases in prostate cancer cell lines by RT-PCR and Tyk2 protein in human prostate cancer specimens by immunohistochemistry. Inhibition of Tyk2 signaling resulted in attenuation of the urokinase-type plasminogen activator-enhanced invasiveness of prostate cancer cells *in vitro* without affecting the cellular growth rate. These results suggest that Tyk2 signaling in prostate cancer cells facilitate invasion of these cells, and interference with this signaling may be a potential therapeutic pathway.
© 2007 Elsevier Inc. All rights reserved.

Keywords: Tyk2; Jak; Prostate cancer; Invasion; Metastasis

Prostate cancer arises as a consequence of an imbalance between cell division and differentiation. The proliferation, differentiation, growth, and apoptosis of normal and malignant cells are regulated by many different cytokines and growth factors. Protein tyrosine kinases (PTKs) play a central role in the proliferation and differentiation of various types of cells. They participate in the cellular responses to growth factors, and activation of their protein kinase activity is critical for the transmission of mitogenic signals. In a previous study, to explore the function of PTKs in the developing prostate gland, we screened for PTKs expressed in CD44-positive cells from the developing mouse prostate. CD44 is a cell surface glycoprotein receptor and this signaling regulates several important biologic processes including lymphocyte homing and activation, hematopoiesis, and tumor progression and metastasis [1]. In addition, CD44 is expressed during mouse prostate development but not in the adult prostate. Treatment with neutralizing antibodies to CD44 inhibits androgen-stimulated ductal branching morphogenesis in serum-free organ cultures of the mouse prostate [2]. Therefore, CD44 is one of the markers of early progenitor cells in prostate tissues. Using CD44 as a cell surface marker, we isolated several PTK genes including Tyk2 from CD44-positive prostate cells [3].

To date, four mammalian members of the Jak family have been identified, namely, Tyk2, Jak1, Jak2, and Jak3. Previous reports from other laboratories have also demonstrated that Jaks are expressed in a human prostate cancer xenograft model and in bone marrow metastases [4,5]. Extensive studies over the last few years have suggested

Abbreviations: Jak, Janus kinase; RT-PCR, reverse transcriptase-polymerase chain reaction; SH2, Src homology 2; Stat, signal transducers and activators of transcription.

* Corresponding author. Fax: +81 3 3964 8934.
E-mail address: shorie@med.teikyo-u.ac.jp (S. Horie).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.08.160

Please cite this article in press as: H. Ide et al., Tyk2 expression and its signaling enhances the invasiveness ..., Biochem. Biophys. Res. Commun. (2007), doi:10.1016/j.bbrc.2007.08.160

that Jak kinases play important roles in the responses to interferons, interleukins, growth factors, hormones, and urokinase-type plasminogen activator (uPA), also known as urokinase [6,7]. Activation of Jaks leads to the tyrosine phosphorylation of receptors, producing docking sites for various SH2-containing signaling molecules including Stat proteins [8]. It is well documented that Stats are over-activated in some malignancies. For example, Stat3 activity is elevated in prostate cancer [9]. Accumulating evidence for constitutive activation of various Stats and other oncoproteins in different cancers strongly suggests that Jak kinases play critical roles in the pathogenesis of many human neoplastic diseases [6].

In this study, we examined the expression and biological significance of Tyk2 in prostate cancer. We show that Tyk2 is involved in uPA-induced cell invasion, which is a measure of the malignant potential of prostate cancer cells. Blockade of Tyk2 signaling by a small interfering RNA (siRNA) or by the PTK inhibitor tyrphostin A1 significantly suppresses the invasiveness of human prostate cancer cells into Matrigel. Our results demonstrate that activation of the Tyk2 signaling pathway is important for the enhancement of prostate cancer cell invasiveness by uPA. Thus, the Tyk2 signaling pathway may be a worthwhile target for therapeutic intervention in prostate cancer.

Materials and methods

Cell culture and reagents. Three human prostate cancer cell lines LNCaP, PC-3, DU145, and TSU-Pr1 (bladder cancer cell line) and MCF-7 (breast cancer cell line) were used in this study. The cells were routinely maintained in RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cell growth was assessed using a colorimetric proliferation assay employing the tetrazolium 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H* tetrazolium (MTS). Each day, MTS was added, and the absorbance at 490 nm was measured on microplate reader after a 60-min incubation at 37 °C. The siRNA duplex targeting Tyk2 and a control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For transfection with siRNAs, DU145 cells were placed in Opti-MEM I (Invitrogen, Carlsbad, CA) and then transfected using oligofectamine (Invitrogen). Tyrphostin A1 (Sigma–Aldrich, St. Louis, MI), an inhibitor of Tyk2 PTK activity, was used at 100 μM, and the effect of uPA (R&D Systems, Minneapolis, MN) was examined at a concentration of 5 nM.

RT-PCR analyses. For RNA expression analysis, total RNA was extracted from cells using RNA Bee (Tel-Test, Friendswood, TX) according to the manufacturer's protocol. The RNA was then treated with DNAse I to remove contaminating DNA and then reverse transcribed using an oligo-dT primer and Super-Scriptβ reverse transcriptase (Invitrogen) in a volume of 25 μl. The primer sequences were as follows: human Jak1, 5′-AAGTGATGTCCTTACCACA-3′ and 5′-AGCAGCCACAC TCAGGTTCT-3′; human Jak2, 5′-GAGCCTATCGGCATGGAATA-3′ and 5′-ATATCTAACACTGCCATCCC-3′; human Jak3, 5′-CAAACAC CACTCCCTGTCCT-3′ and 5′-TGGGGGTGTTCCTGAAGTAG-3′; Tyk2, 5′-GGATGGCCAGGGGCAGTAAG-3′ and 5′-GGATCTCCTC CTCGGTCGAC-3′; prostate-specific antigen, 5′-GGTCGGCACAGCC TGTTTCA-3′ and 5′-CCACGATGGTGTCCTTGATC-3′; β-actin, 5′-GACTACCTCATGAAGATCCT-3′ and 5′-GCGGATGTCCACGTCA CACT-3′. The resulting cDNA was subjected to PCR.

Immunoblot analyses. The cells were washed twice with cold PBS, and lyzed on ice in 2× sample buffer (125 mmol/L Tris, pH 6.8, 4% SDS, 10% 2-β mercaptoethanol, 20% glycerol, 0.06% bromophenol blue). The cell lysates were boiled for 3 min and resolved by 10% SDS–PAGE. Proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA), and immunoblotting was performed using rabbit anti-human Tyk2 antibody (Santa Cruz Biotechnology; 1:1000) or rabbit anti-human β-tubulin antibody (Santa Cruz Biotechnology) as an internal loading control. Goat anti-rabbit antibody conjugated by HRP (Bio-Rad, 1:3000 dilution) were used as a secondary antibody. Immunoreactive proteins were visualized with ECL detection reagents (Amersham Biosciences, Piscataway, NJ).

Immunohistochemistry. Serial 4-mm-thick sections were deparaffinized in three changes of xylene and rehydrated through a graded series of ethanol decreasing from 100% to 70%. The sections were immersed in citrate buffer (pH. 6.0) and autoclaved at 120 °C for 5 min and then placed in 3% hydrogen peroxide in methanol for 20 min at room temperature to block endogenous peroxidase activity. Nonspecific protein binding was blocked by incubating the section for 30 min–1 h in 5% goat serum. Next, the sections were incubated overnight at 4 °C in polyclonal rabbit Tyk2 antibody (Santa Cruz Biotechnology). Sections were then processed for immunohistochemistry using the EnVisionTM+ system (DAKO, Denmark). We examined 70 samples from prostate cancer patients.

Matrigel invasion assay. Membrane inserts (8-μm pore size) for 24-well transwell plates were prepared by coating with Matrigel basement membrane matrix (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. DU145 cells were placed in the upper chamber at a density of 2×10^4 cells/insert. Medium containing 5 nM uPA (R&D Systems, Minneapolis, MN) was added to the lower chamber as a chemoattractant. To inhibit the invasion, 200 nM of Tyk2 siRNA/200 nM oligofectamine or 100 μM of tyrphostin A1 was added to the medium. After 24 h, the upper surface of the inserts was wiped with cotton swabs, and the inserts were stained with Trypan blue. Cells that migrated through the Matrigel and the filter pores to the lower surface were counted in five random high-power fields per insert using a light microscope.

Results

Expression analyses of Tyk2 in prostate cancer cell lines and tissues

We first analyzed the expression of Jaks in MCF-7, LNCaP, PC-3, TSU-Pr1 and DU145 cells by RT-PCR (Fig. 1A). Amplified products for four members of the Jak family, Tyk2, Jak1, Jak2, and Jak3, were clearly detected in these cell lines. We also found that prostate-specific antigen was expressed by LNCaP cells which is androgen sensitive prostate cancer cell line as previously described [3]. Although we tested one androgen sensitive prostate cancer cell line LNCaP, there was no difference of the expression level of Jaks mRNAs between androgen sensitive cells and insensitive cells. We next examined the expression of Tyk2 in human prostate cancer tissue specimens by immunohistochemistry with rabbit anti-human Tyk2 antiserum. The reactivity for Tyk2, shown as brown color, was higher in cancerous than in noncancerous glands in the same field (Fig. 1B). The staining for Tyk2 was heterogeneous and predominately located in the cytoplasm of prostate cancer cells. Of 70 samples from prostate cancer patients, Tyk2 staining was clearly detected in 17 (24.3%). Further studies are needed to accurately determine the correlation between Tyk2 expression in the cancerous tissues and clinical and pathological variables.

Please cite this article in press as: H. Ide et al., Tyk2 expression and its signaling enhances the invasiveness ..., Biochem. Biophys. Res. Commun. (2007), doi:10.1016/j.bbrc.2007.08.160

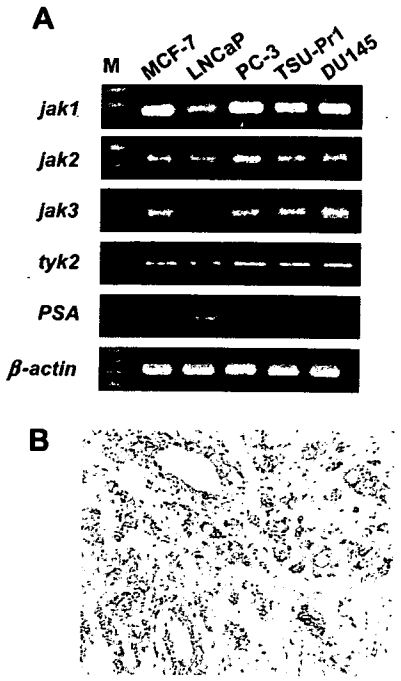


Fig. 1. (A) RT-PCR analysis of Jak family kinases mRNA levels in prostate cancer cell lines. MCF-7 is a breast cancer-derived cell line. (B) Immunohistochemical analysis of Tyk2 expression in prostate cancer tissues. Strong staining is observed in cancerous cells. Histopathological evaluation was done by a pathologist (Y.T.) and hematoxylin and eosin staining shows malignant features of these cells (data not shown).

Interference of Tyk 2 signaling dose not inhibit proliferation of prostate cancer cells

We next examined the role of Tyk2 in cell proliferation using a 96-well-based MTS assay. The experiments were carried out with cells at several different passages and with different batches of transfected cells. At a concentration of 100 μM, tyrphostin A1, a tyrosine kinase inhibitor, did not inhibit the growth rate of DU145 cells (Fig. 2A). Down-regulation of Tyk2 expression using a siRNA also did not inhibit the growth rate of DU145 cells, even though the expression of Tyk2 was decreased (Fig. 2B). These results suggest that signaling by Tyk2 does not affect the proliferation of DU145 cells. Finally, uPA did not affect the growth rate of DU145 cells (Fig. 2C).

Tyk2 signaling involved in invasiveness of prostate cancer cells through uPA

Recent reports suggest that Tyk2 regulates cell migration by mediating uPA activation of phosphatidylinositol 3-kinase [10]. Although uPA is known to regulate the invasiveness of prostate cancer cells, the underlying molecular mechanisms are still unclear. To determine whether Tyk2 participates in the promotion of invasiveness by uPA, we performed Matrigel invasion chamber assays. DU-145 cells were placed in transwells containing Matrigel-coated porous

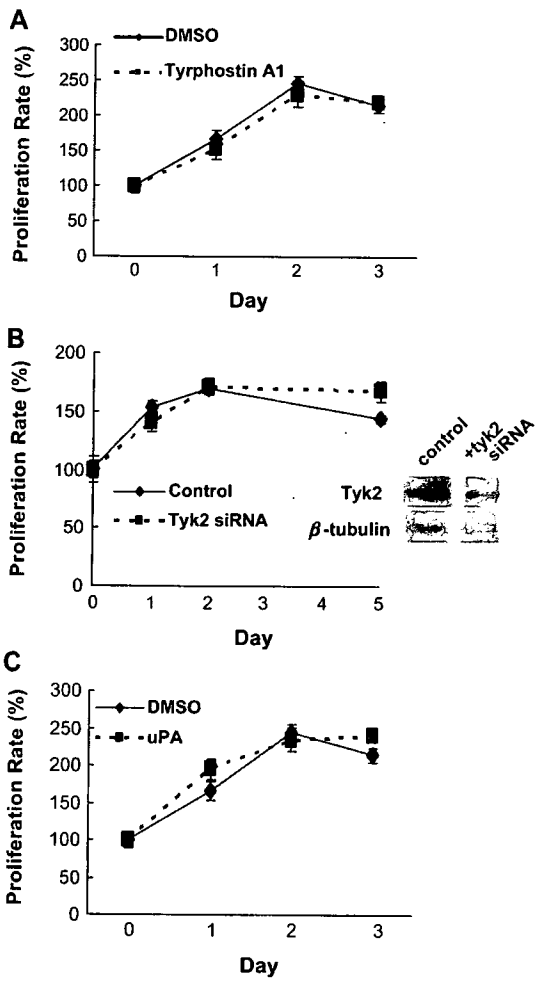


Fig. 2. Role of Tyk2 signaling in the growth of DU145 cells. (A) Tyrphostin A1 did not affect the growth of DU145 cells. (B) The siRNA targeting Tyk2 also did not affect the growth of DU145 cells. (C) uPA did not change a proliferation rate of DU-145 cells. The number of live cells was determined by MTS assay. These assays were repeated two times with different batches of transfected cells, and each cell sample was done in triplicate.

membranes, and the lower chamber was filled with medium with or without uPA. After 24 h, cells that migrated through the Matrigel basement membrane matrix and the filter pores to the lower surface of the membrane were counted by light microscopy. Treatment of cells with tyrphostin A1 inhibited the invasiveness in the presence or absence of uPA in the lower chamber of the transwell (Fig. 3A). In addition, cells transfected with the Tyk2 siRNA, showed decreased invasiveness compared to cells transfected with control siRNA (Fig. 3B). The results with the Tyk2 inhibitor and using the siRNA indicated that inhibition of Tyk2 signaling reduces the invasiveness of prostate cancer cells *in vitro*.

Discussion

In prostate cells, transformation, tumorigenesis, and metastasis can be caused by alterations in cellular regulation,

Please cite this article in press as: H. Ide et al., Tyk2 expression and its signaling enhances the invasiveness ..., Biochem. Biophys. Res. Commun. (2007), doi:10.1016/j.bbrc.2007.08.160

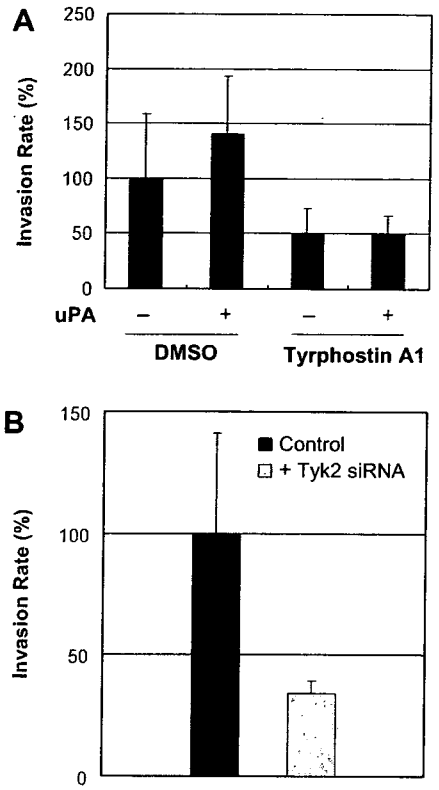


Fig. 3. The enhancement of invasion by uPA is suppressed by blockade of Tyk2 signaling in DU-145 cells. (A) The enhancement of invasiveness by uPA was suppressed by tyrphostin A1, an inhibitor of Tyk2 phosphorylation, in DU145 cells. (B) Down-regulation of Tyk2 expression with a siRNA inhibited the invasiveness of uPA-treated DU145 cells. Assays examining the effect of siRNA were repeated three times with different batches of transfected cells.

especially by dysregulation of signaling by PTKs, including receptors for transforming growth factorα, epidermal growth factor, insulin-like growth factor 1, fibroblast growth factors, hepatocyte growth factor, platelet-derived growth factor, nerve growth factors, and interleukin-6 [11]. For example, immunocytochemical analysis has shown that platelet-derived growth factor receptor-α is expressed in bone marrow metastases from prostate tumors [4]. Also, overexpression of HER-2/neu in primary prostate cancer and in metastatic sites of prostate tumors has been detected before and after hormone therapy [12]. In addition, in prostate cancer cells, interleukin-6 enhances cell growth and causes a parallel activation of the Stat3 signaling pathway [13]. Although some of the signaling components have been investigated in prostate cancer cells, only a few of these PTK genes have been shown to influence their invasiveness and metastatic potential.

CD44 has been implicated in a number of important biologic processes, including lymphocyte homing and activation, hematopoiesis, and tumor progression and metastasis [14]. CD44 may be a marker for immature and progenitor cells in prostate tissues. We previously identified Jak1 and Tyk2 using a PCR-based strategy to screen for PTKs in CD44-positive prostate cells [3]. To our knowledge, this is the first report that Tyk2 plays a significant role in mediating the enhancement of prostate cancer invasion by uPA. We found that prostate cancer cells express Jak family kinases, and in the current studies, we concentrated the role of Tyk2. Blockade of Tyk2 expression using a siRNA significantly suppressed the promotion of human prostate cancer cell invasiveness by uPA. In addition, the Tyk2 inhibitor tyrphostin A1 suppressed the invasiveness of human prostate cancer cells. We also showed that tissue samples from prostate cancer patients are positive for Tyk2.

The activation of Tyk2 signaling may depend on upstream events such as the activation of the uPA, interleukin, and interferon receptors. Furthermore, uPA is a multifunctional molecule that acts as both a proteolytic enzyme and a ligand that induces intracellular signaling. The uPA receptor mediates intracellular signaling *via* surface proteins such as integrins, growth factors receptors, and G-protein-coupled membrane proteins [15]. Several reports have shown that Jaks and Stats interfere with multiple signaling cascades, such as the Ras/mitogen-activated protein kinase pathway and activation of phosphatidylinositol 3-kinase and Src kinases [16]. For instance, Tyk2 is required for the activation of Stat3 by uPA in glomerular mesangial cells [17]. In human vascular smooth muscle cells, uPA stimulates migration *via* the uPA receptor signaling complex, which contains the Tyk2 and phosphatidylinositol 3-kinase [10]. Together with these previous findings, our results suggest that Tyk2 is one of the key molecules in mediating uPA receptor signaling in prostate cancer cells.

Our results also suggest that Tyk2 signaling may contribute to the metastasis of prostate cancer. Further studies are needed to determine whether inhibition of Tyk2 attenuates the metastasis of prostate cancer cells in *in vivo*. We are currently examining the correlation between the expression of Tyk2 in prostate cancer and the pathological and clinical variables. Several inhibitors of Jak protein kinase have been developed as molecular-targeted chemotherapeutic agents [18]. Jak kinase generally functions as a modulator at the intersection of multiple signal transduction pathways. Thus, targeting Tyk2 with specific drugs may be useful for therapeutic intervention in prostate cancer.

References

[1] D. Naor, R.V. Sionov, D. Ish-Shalom, CD44: structure, function, and association with the malignant process, Adv. Cancer Res. 71 (1997) 241–319.
[2] P. Gakunga, G. Frost, S. Shuster, G. Cunha, B. Formby, R. Stern, Hyaluronan is a prerequisite for ductal branching morphogenesis, Development 124 (1997) 3987–3997.
[3] H. Ide, D.B. Seligson, S. Memarzadeh, L. Xin, S. Horvath, P. Dubey, M.B. Flick, B.M. Kacinski, A. Palotie, O.N. Witte, Expression of colony-stimulating factor 1 receptor during prostate development and prostate cancer progression, Proc. Natl. Acad. Sci. USA 99 (2002) 14404–14409.
[4] A. Chott, Z. Sun, D. Morganstern, J. Pan, T. Li, M. Susani, I. Mosberger, M.P. Upton, G.J. Bubley, S.P. Balk, Tyrosine kinases expressed in vivo by human prostate cancer bone marrow metastases

Please cite this article in press as: H. Ide et al., Tyk2 expression and its signaling enhances the invasiveness..., Biochem. Biophys. Res. Commun. (2007), doi:10.1016/j.bbrc.2007.08.160

and loss of the type 1 insulin-like growth factor receptor, Am. J. Pathol. 155 (1999) 1271–1279.
[5] D. Robinson, F. He, T. Pretlow, H.J. Kung, A tyrosine kinase profile of prostate carcinoma, Proc. Natl. Acad. Sci. USA 93 (1996) 5958–5962.
[6] A. Verma, S. Kambhampati, S. Parmar, L.C. Platanias, Jak family of kinases in cancer, Cancer Metastasis Rev. 22 (2003) 423–434.
[7] D. Hebenstreit, J. Horejs-Hoeck, A. Duschl, JAK/STAT-dependent gene regulation by cytokines, Drug News Pers. 18 (2005) 243–249.
[8] K. Yamaoka, P. Saharinen, M. Pesu, V.E. Holt 3rd, O. Silvennoinen, J.J. O'Shea, The Janus kinases (Jaks), Genome Biol. 5 (2004) 253.
[9] J.V. Alvarez, P.G. Febbo, S. Ramaswamy, M. Loda, A. Richardson, D.A. Frank, Identification of a genetic signature of activated signal transducer and activator of transcription 3 in human tumors, Cancer Res. 65 (2005) 5054–5062.
[10] A. Kusch, S. Tkachuk, H. Haller, R. Dietz, D.C. Gulba, M. Lipp, I. Dumler, Urokinase stimulates human vascular smooth muscle cell migration via a phosphatidylinositol 3-kinase-Tyk2 interaction, J. Biol. Chem. 275 (2000) 39466–39473.
[11] D. Djakiew, Dysregulated expression of growth factors and their receptors in the development of prostate cancer, Prostate 42 (2000) 150–160.
[12] I. Osman, H.I. Scher, M. Drobnjak, D. Verbel, M. Morris, D. Agus, J.S. Ross, C. Cordon-Cardo, HER-2/neu (p185neu) protein expression in the natural or treated history of prostate cancer, Clin. Cancer Res. 7 (2001) 2643–2647.
[13] Z. Culig, G. Bartsch, A. Hobisch, Interleukin-6 regulates androgen receptor activity and prostate cancer cell growth, Mol. Cell Endocrinol. 197 (2002) 231–238.
[14] D. Naor, S. Nedvetzki, I. Golan, L. Melnik, Y. Faitelson, CD44 in cancer, Crit. Rev. Clin. Lab. Sci. 39 (2002) 527–579.
[15] F. Blasi, P. Carmeliet, uPAR: a versatile signalling orchestrator, Nat. Rev. Mol. Cell Biol. 3 (2002) 932–943.
[16] A.C. Ward, I. Touw, A. Yoshimura, The Jak–Stat pathway in normal and perturbed hematopoiesis, Blood 95 (2000) 19–29.
[17] N. Shushakova, N. Tkachuk, M. Dangers, S. Tkachuk, J.K. Park, J. Zwirner, K. Hashimoto, H. Haller, I. Dumler, Urokinase-induced activation of the gp130/Tyk2/Stat3 pathway mediates a pro-inflammatory effect in human mesangial cells via expression of the anaphylatoxin C5a receptor, J. Cell Sci. 118 (2005) 2743–2753.
[18] J.E. Thompson, JAK protein kinase inhibitors, Drug News Pers. 18 (2005) 305–310.

Please cite this article in press as: H. Ide et al., Tyk2 expression and its signaling enhances the invasiveness ..., Biochem. Biophys. Res. Commun. (2007), doi:10.1016/j.bbrc.2007.08.160