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Stimulation of cellular senescent processes, including secretory phenotypes and anti-oxidant responses, after androgen deprivation therapy in human prostate cancer

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ABSTRACT

Endocrine resistance is a major problem in prostate cancer. Recent studies suggest that cellular plasticity plays a key role in therapy resistance. Yet little is known about the cellular changes of human prostate cancer after androgen deprivation therapy (ADT). In this study, we investigated cellular senescence, senescence-associated secretory phenotypes (SASPs), and anti-oxidant responses. Hormone ablation upregulated senescence-associated (SA)-β-Gal activity in prostate glands, as well as the expressions of $p27^{KIP1}$ and p53, in a mouse castration model. In line with this, the expressions of $p21^{CIP1}$ and $p27^{KIP1}$ were significantly more upregulated in human non-pathological prostatic glands after ADT than in untreated specimens. In a study of SASP markers, the expressions of IL6 and IL8 were also more upregulated in human non-pathological prostatic glands after ADT than in untreated specimens. IL6, IL8, and MMP2 were expressed more strongly in human prostate cancer specimens resected after ADT than in untreated tumors. Of note, treatment with the anti-oxidant reagent NAC significantly suppressed SA-β-Gal activity in androgen-sensitive human prostate cancer LNCaP cells. In immunohistochemical analyses on anti-oxidant response genes, NRF2 and NQO1 were more upregulated after hormone ablation in human prostate gland and carcinoma specimens after ADT than in untreated specimens or in murine prostate glands after castration. Taken together, these findings suggest that ADT induces cellular senescence processes accompanied by secretory phenotypes and anti-oxidant responses in prostate. These cellular changes may be attractive targets for preventing endocrine resistance in prostate cancer.

1. Introduction

Prostate cancer is one of the most common cancers in men around the world. An androgen receptor (AR)-mediated pathway generates a pivotal axis in the cell proliferation of prostate cancer, and androgen-deprivation therapy (ADT) has been widely accepted as the first line of treatment [1]. The tumors, however, become refractory to ADT due to the expansion of hormone-unresponsive tumor populations. The molecular mechanisms underlying the hormone-unresponsive progression of prostate cancer remain largely unraveled in spite of intensive investigations [2,3]. Recent reports on general cancers suggest that cellular plasticity and diversity contribute to therapy resistance [4,5]. An earlier study by our group showed that cellular plasticity may be wide-ranging in prostate cancer by demonstrating that epithelial-mesenchymal transition (EMT), a type of cellular plasticity, takes part in the hormone-unresponsive progression of prostate cancer [6]. In this study we extended our analysis of cellular plasticity to cellular senescence, senescence-associated secretory phenotypes, and anti-oxidant responses under androgen deprivation.

Cellular senescence is defined as a permanent cell-cycle-arrest triggered by various stresses, including telomere shortening and DNA damages [7]. Accumulating evidence indicates that cellular senescence is tightly linked to tumorigenesis [8,9]. Over-stimulation of oncogenic Ras, for example, triggers oncogene-induced senescence, a barrier for tumorigenesis [10]. Cellular senescence is

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also reported to protect against the tumor formation of human melanocytic nevi, as well as tumor formation in murine models of liver cancer and lymphoma [11–13]. Other notable reports have shown that cellular senescence stimulates inflammatory cytokine secretion [14–18]. These senescence-associated secretory phenotypes (SASPs) may contribute to the progression of various cancers via their established effects in accelerating both tumor growth and invasion [19]. Little is known, however, about the involvement of SASPs in clinical human prostate cancer. In this study we demonstrated that senescence-associated β-Gal (SA-β-Gal) activity is upregulated in the prostatic glands of castrated mice. We were also interested to find that treatment with an anti-oxidant reagent, *N*-acethyl-L-cystein (NAC), suppressed SA-β-Gal activity after androgen ablation in androgen-sensitive human prostate cancer LNCaP cells. This is intriguing, as it suggests that cellular senescence phenotypes are mediated in part through an increase of reactive oxygen species (ROS). An immunohistochemical analysis showed that SASP markers and anti-oxidant response molecules are upregulated more markedly in human prostatic glands and prostate cancer cells resected after ADT than in untreated glands and tumors. Taken together, the evidence suggests that cellular senescence and its associated responses may be involved in the hormone-unresponsive progression of prostate cancer.

2. Materials and methods

2.1. Animals

ICR mice were purchased from Japan SLC (Shizuoka, Japan). Ten-week-old male and female ICR mice were castrated by either orchiectomy or ovariectomy (day 0), and sacrificed on day 7. Proteins, RNAs and tissue sections were prepared from various tissues (n = 3–5 per each group). Sham-operated mice were used as controls (n = 3–5 per each group). Three independent animal experiments were performed. The experimental protocols were approved by the local committee for animal experimentation at Jichi Medical University.

2.2. Cases

Human prostate adenocarcinoma specimens from 30 cases resected after ADT and 30 cases resected without pretreatment were retrieved from the archives of the Pathology Department of Jichi Medical University Hospital (10 specimens in each category were also used in our previous study) [6]. The patient characteristics are summarized in Supplementary Table A1. The duration of ADT was 1–27 months (mean; 5.37 months). Non-pathological prostatic glands of these cases were also analyzed. Immunohistochemical studies were performed with the approval of the local ethics committee at Jichi Medical University.

2.3. Cells and reagents

Androgen-sensitive human prostate cancer LNCaP cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). NAC and β-nicotinamide mononucleotide (NMN) were purchased from Sigma-Aldrich (St. Louis, MO).

2.4. SA-β-Gal assay and TUNEL assay

SA-β-Gal staining was performed using a senescence detection kit (BioVision, Mountain View, CA). Frozen sections from castrated and sham-operated mice were washed twice with PBS and incubated overnight with a X-Gal-containing staining solution at 37 °C. The positive cells were counted in four different areas. Apoptosis was evaluated by a TUNEL assay with an ApopTag In Situ Apoptosis Detection Kit (CHEMICON International, Temecula, CA) using a previously described method [20]. Briefly, paraffin-embedded sections from castrated and sham-operated mice were deparaffinized, and nucleotide nick ends were tailed with digoxigenin-dNTPs using terminal deoxynucleotidyl transferase (TdT) for 1 h at 37 °C. The sections were then incubated with an anti-digoxigenin antibody conjugated to peroxidase, and immunostained with 3,3′-diaminobenzidine tetrahydrochloride. The number of positive cells per 100 cells were counted in four different areas on each section.

LNCaP cells (5×10^4 cells/well) were plated onto 4-well chamber slides in a phenol-red-free RPMI 1640 medium with 2% FBS treated with dextran-coated charcoal (dcc). The next day (day 0), the medium was changed to a phenol-red-free RPMI 1640 medium with 2% dcc-treated FBS in the presence or absence of 1 mM NAC, 100 μM NMN, and 10 nM testosterone. The medium was changed every other day. On day 4, the slide chamber was similarly stained and the positive cells were counted.

2.5. Immunoblots

Tissue extracts were prepared from frozen sections of murine prostate using a cell lysis reagent of CelLytic MT (Sigma-Aldrich). Ten micrograms of tissue extracts were separated by SDS-PAGE electrophoresis and transferred onto PVDF membranes. The blots were reacted with the antibodies listed in Table 1 and the signals were detected with an ECL prime kit (GE Healthcare, Buckinghamshire, UK). The signal intensity was monitored by ImageJ software. For loading controls, the membranes were stripped with reprobe buffer [62.5 mM Tris–HCl (pH6.8), 100 mM 2-mercaptoethanol, 2% SDS] at 60 °C for 30 min and then immunoblotted with an anti-GAPDH antibody (MILLIPORE, Temecula, CA) followed by the secondary antibody.

2.6. Immunohistochemistry and antibodies

Table 1 summarizes the antibodies used in this study and the methods for retrieving and detecting the antigens. In general, formalin-fixed and paraffin-embedded sections were pre-treated in a microwave oven or an autoclave for the indicated times. The pre-treated sections were then allowed to interact with each

Table 1
Immunohistochemical protocols.

antibodies		dilution ratio	pretreat
$p16^{INK4a}$	mouse monoclonal (Abcam, Cambridge MA)	1:200	Autoclave 5 min
$p21^{CIP1}$	mouse monoclonal (Santa Cruz, Dallas TX)	1:20	Autoclave 5 min
$p27^{KIP1}$	mouse monoclonal (BD Transduction, Dallas TX)	1:50	Microwave 20 min
p53	mouse monoclonal (Cell signaling, Danvers MA)	1:100	Microwave 10 min
IL6	rabbit polyclonal (Abcam, Cambridge MA)	1:800	Microwave 10 min
IL8	rabbit polyclonal (Abcam, Cambridge MA)	1:800	Microwave 10 min
MMP2	mouse monoclonal (Abcam, Cambridge MA)	1:400	Microwave 10 min
MMP9	mouse monoclonal (Abcam, Cambridge MA)	1:800	Microwave 10 min
NRF2	rabbit monoclonal (MBL, Woburn MA)	1:1600	Microwave 20 min
NQO1	mouse monoclonal (Cell signaling, Danvers MA)	1:100	Autoclave 5 min
SQSTM1	rabbit polyclonal (MBL, Woburn MA)	1:1000	Microwave 20 min