Prospective Identification of Tumorigenic Prostate Cancer Stem Cells

Anne T. Collins, Paul A. Berry, Catherine Hyde, Michael J. Stower, and Norman J. Maitland

Abstract

Existing therapies for prostate cancer eradicates the bulk of cells within a tumor. However, most patients go on to develop androgen-independent disease that remains incurable by current treatment strategies. There is now increasing evidence in some malignancies that the tumor cells are organized as a hierarchy originating from rare stem cells that are responsible for maintaining the tumor. We report here the identification and characterization of a cancer stem cell population from human prostate tumors, which possess a significant capacity for self-renewal. These cells are also able to regenerate the phenotypically mixed populations of nonclinical malignancies, which express differentiated cell products, such as androgen receptor and prostatic acid phosphatase. The cancer stem cells have a CD44+/α2β1hi/CD133+ phenotype, and we have exploited these markers to isolate cells from a series of prostate tumors with differing Gleason grade and metastatic states. Approximately 0.1% of cells in any tumor expressed this phenotype, and there was no correlation between the number of CD44+/α2β1hi/CD133+ cells and tumor grade. The identification of a prostate cancer stem cell provides a powerful tool to investigate the tumorigenic process and to develop therapies targeted to the stem cell. (Cancer Res 2005; 65(23): 10946-51)

Introduction

Considerable research efforts have been directed toward the identification of markers associated with the initiation and progression of prostate cancer; yet, there is little consensus about the target cell within prostate epithelium that is susceptible to malignant transformation or about the mechanism that underlies the heterogeneity of prostate tumors. Prostate tumors are particularly heterogeneous (i.e., composed of cells with different phenotypic characteristics and different proliferative and malignant potentials; refs. 1, 2). In leukemia and some solid cancers, only a small subset of cells is clonogenic in culture and in vivo (3, 4). This led to the hypothesis that only a rare, phenotypically distinct subset of cells has the capacity to form new tumors and that this subgroup could be considered as cancer stem cells (4). To prove this hypothesis, subsets of uncultured cancer cells, enriched for the ability to form new tumors, would have to be purified. This was accomplished by Bonnet and Dick (5) who showed that a small proportion of human acute myeloid leukemia (AML) cells expressed the stem cell phenotype CD34+ and that only these cells were able to transfer AML to immunodeficient mice. Since this seminal article, similar studies have shown that subpopulations of tumor cells from breast (6), brain (7, 8), and lung (9) have stem cell characteristics.

We have pioneered the identification and isolation of stem cells from human prostate epithelium based on high surface expression of integrin α2β1 (10) and CD133 (11). As tumor cells might be expected to share some of their antigenic properties with tissue stem cells from the same organ (5), we have applied the same techniques used to isolate normal prostate epithelial stem cells to the analysis of the tumorigenic cells in human prostate cancers. We report here the identification and characterization of cells from primary and metastatic prostate tumors that have cancer stem cell characteristics, namely a capacity for extensive proliferation, self-renewal, differentiation, and invasion.

Materials and Methods

Tissue collection, isolation, and culture of tumor stem cells. Human prostate tissue was obtained, with patient consent, from 40 patients (age range 56-69 years) undergoing radical prostatectomy for prostate cancer. Prostate cancer was confirmed by histologic examination of representative adjacent fragments. In some cases, lymph node biopsies were taken if metastasis was suspected. CD44+/α2β1hi/CD133+ cells were isolated from the tissue, as described previously for normal prostate epithelium (11), and were subsequently placed in long-term culture from four tumor samples and one benign biopsy. Cultures were maintained in complete keratinocyte growth medium [keratinocyte serum-free medium with epidermal growth factor (EGF) and bovine pituitary extract; Invitrogen, Paisley, Scotland]. The medium was also supplemented with 2 ng/mL of leukemia inhibitory factor (LIF; Sigma, Poole, United Kingdom), 2 ng/mL of stem cell factor (Sigma), and 100 ng/mL of cholera toxin (Sigma).

Cultures were also derived from two lymph node metastasis of the prostate but initially from an unselected epithelial population. However, after expansion in culture, CD44+/α2β1hi/CD133+ were isolated and cultures were derived.

Coloncy-forming and long-term serial culture assays. The potential of the tumor populations, CD44+/α2β1hi/CD133+ (stem cells), CD44+/α2β1hi/CD133+ (transit amplifying population), and CD44+/α2β1low (committed basal population), to form colonies was compared with the unselected tumor population. Briefly, the above phenotypes were counted and plated on collagen-coated (BD-Biocoat) plates (BD Biosciences, Oxford, United Kingdom) for the determination of both colony-forming efficiency and self-renewal. Colonies were counted, ring-cloned (after 28 days if they contained >32 cells), and replated into individual wells of a 24-well plate. As the number of cells selected was small, irradiated (60 Gy) STO (mouse embryonic fibroblasts) cells were added as feeders. For the long-term serial culture assays, used to determine the proliferative potential of selected phenotypes, cells were passaged continuously. At each passage, the number of cells generated by each fraction was determined. The cumulative total cell output from the initial 10^3 cells was calculated at the end of the experiment, assuming that all cells from each passage had been replated.
Anchorage-independent growth was also assessed in methylcellulose (Miltenyi Biotec Ltd., Bisley, Surrey, United Kingdom). Briefly, the above phenotypes were placed in complete methylcellulose (supplemented with EGF, bovine pituitary extract, stem cell factor, ILF, and cholera toxin at the concentrations used for complete keratinocyte serum-free medium) and the number of surviving cells was counted after 28 days. The results were subjected to a paired t test to determine statistical significance.

**Immunofluorescent staining of CD44+/α3β1hi/CD133+ cells.** CD44+/α3β1hi/CD133+ cells were selected directly from disaggregated tumor tissue or cultured cells from tumors before processing for dual-color imaging under confocal microscopy by fixation in ice-cold methanol for 20 minutes and permeabilization in 0.4% Triton X-100 (Sigma) containing 0.3% normal goat serum (NGS) for 10 minutes. After blocking (1:5 dilution of NGS in TBS for 10 minutes), cells were incubated with antibodies against keratins 1, 5, 10, and 14 (clone 34E12, DakoCytomation, Ely, Cambridge, United Kingdom); keratin 5 (clone RCK103, BD PharMingen, Oxford, England); keratin 14 (clone LL002, Serotec Ltd., Oxford, England); and keratin 19 (clone RCK 108, DakoCytomation); keratin 18 (clone CY-90; Sigma); c-Met (clone 8F11, Vector Laboratories, Inc., Peterborough, United Kingdom); prostatic acid phosphatase (clone PASE/4LJ, DakoCytomation); androgen receptor (clone AR27, Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom); keratin 5 (clone RCK103, BD PharMingen, Oxford, England); and prostate-specific stem cell antigen (clone B3299, kindly donated by Genentech, Inc., San Francisco, CA) or a nonspecific isotype control for 1 hour. Appropriate positive control cells were stained in parallel for each antibody used for CD133+ cell phenotyping. After washing (3× TBS), cells were further probed with FITC-tagged secondary antibody. Cells were mounted in the antiphotobleaching medium Vectashield containing 4,6-diamidino-2-phenylindole (DAP; Vector Laboratories).

**Invasion activity assay in Matrigel.** The ability of epithelial cells to migrate through Matrigel was determined by the modified Boyden-chamber method (12). Briefly, tumor cells, derived initially from CD133+-selected populations from patients with prostate cancer, were plated onto Matrigel-coated 8 µm filters. Conditioned medium from prostate stromal cultures (13) was used as a chemoattractant. Cells invading through Matrigel and filters were counted 48 hours after plating. Epithelial cells from benign prostate and PNT1a (an immortalized cell line from benign prostate) were used as negative controls. The positive controls were PC3M (metastatic prostate cancer cell line) and MCF7 (metastatic breast cancer cell line). The results were subjected to a paired t test to determine statistical significance.

**Differentialen of adult CD44+/α3β1hi/CD133+ isolated from prostate tumors.** CD44+/α3β1hi/CD133+ cells were isolated from tumor biopsies and cultured for up to 21 days in RPMI 1640 (Invitrogen), supplemented with 10% FCS and 10−9 mol/L dihydrotestosterone. Cells were subsequently processed for dual-color staining flow cytometry. Dual staining was done on cells fixed and permeabilized in 1% saponin (Sigma). Cells were separated on a DakoCytomation Cyan high-performance flow cytometer and analyzed using DakoCytomation Summit version 3.3 software (DakoCytomation).

**Genotype analysis of tumor cells.** To confirm the identity of the cultures from primary tissues, 40 ng of DNA extracted from the cultured cells derived by CD133 selection was compared with DNA extracted from peripheral blood lymphocytes (PBL) taken from the same patient (14). In some cases, a third comparison, with DNA extracted from serial frozen tissue sections, was also used. Primers spanning five highly polymorphic microsatellites (D8S262, D8S264, D8S560, D8S592, and D16S522), one of which was labeled with a Beckman dye (D2, D3, or D4), were used in a PCR as previously described (14). Touchdown PCR to take account of varying base pair composition was used (10 minutes at 94°C; followed by 16 touchdown cycles of 94°C/45 seconds, 60-52°C/45 seconds, 72°C/30 seconds; then 16 cycles of 94°C/45 seconds, 52°C/45 seconds, 72°C/30 seconds; and finally 72°C/7 minutes). Analysis of the labeled fragments was accomplished on a Beckman Coulter CEQ8000 and proprietary CEQ fragment analysis software to accurately size and quantify alleles.

**Results**

Tumor CD44+/α3β1hi/CD133+ cells exhibit a high proliferative potential in vitro. We have previously established that the phenotype α3β1hi/CD133+ determines normal prostate epithelial stem cells (11). To establish whether α3β1hi/CD133+ cells were present in prostate tumors, we initially used MACS bead sorting to determine the proportion of CD133-expressing cells in a series of 40 prostate tumors. Regardless of the tumor grade, a small population (ranging from 0.1% to 0.3%) of CD133-expressing cells could be isolated (results not shown) from all tumors tested.

To determine whether tumor α3β1hi/CD133+ cells were distinct from the bulk population of cancer cells, we next looked at the proliferative properties of six independently derived tumor cultures in vitro. Long-term cultures were established from four primary prostate tumors and two metastatic prostate tumors from this larger series (Table 1). Self-renewal of specific phenotypes was measured by replating primary colonies and subsequently measuring secondary colony-forming efficiency. As shown in Fig. L4, the CD44+/α3β1hi/CD133+ cells gave rise to 3.7-fold greater numbers of colonies than the total population and this number was significantly greater (P < 0.05) than those formed from either CD44+/α3β1hi/CD133– or CD44−/α3β1low cells. Indeed, 30-fold fewer colonies were formed by the CD44+/α3β1low population compared with the CD133–-selected population. There was no significant difference (P > 0.05) between the primary and secondary colony-forming efficiency of the stem cell population, whereas the ability of the transit amplifying population (CD44+/α3β1hi/CD133−) to undergo self-renewal (secondary colony-forming efficiency) was significantly lower than the stem cell population (P < 0.005). Few primary colonies were formed by CD44+/α3β1low cells and the majority was abortive after one to three rounds of cell division. The ability of this population to form secondary colonies was also significantly less than the stem cell population (P < 0.005; 44-fold less secondary colonies founded). Primary colonies were not founded by the secretory luminal (CD44−/CD57+) population.

To show whether CD44+/α3β1hi/CD133+ cells were capable of extensive proliferation (e.g., generating progeny several orders of magnitude higher that the starting population), cultures were generated from a series of prostate primary and metastatic tumors from a starting population of 103 CD44+/α3β1hi/CD133+ cells (Fig. 1B). The data clearly indicate that tumor cultures derived from CD133+ cells have an enhanced proliferative potential. In contrast, the proliferative potential of CD133− cells, derived from benign prostate, was much lower and proliferation ceased after ~12 PD.

Quantification of CD133 expression, by MACS cell sorting, in long-term cultures of prostate cancer cells ranged from 0.3% to 1.6% (Table 2). Regardless of time in culture, the proportion of

**Table 1. Summary of patient population**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (y)</th>
<th>Origin</th>
<th>Gleason score</th>
</tr>
</thead>
<tbody>
<tr>
<td>228</td>
<td>—</td>
<td>Metastasis</td>
<td>7</td>
</tr>
<tr>
<td>352</td>
<td>69</td>
<td>Prostate</td>
<td>Benign</td>
</tr>
<tr>
<td>434</td>
<td>59</td>
<td>Prostate</td>
<td>8/9</td>
</tr>
<tr>
<td>484</td>
<td>69</td>
<td>Prostate</td>
<td>7</td>
</tr>
<tr>
<td>563</td>
<td>64</td>
<td>Prostate</td>
<td>7</td>
</tr>
<tr>
<td>569</td>
<td>67</td>
<td>Prostate</td>
<td>8</td>
</tr>
<tr>
<td>605</td>
<td>56</td>
<td>Metastasis</td>
<td>7</td>
</tr>
</tbody>
</table>

NOTE: All metastases were from local lymph nodes.
CD133-expressing cells remained relatively constant and did not differ significantly from the freshly isolated population \( (P > 0.05) \).

**Tumor cell origins of the in vitro cultures.** To confirm that we had derived CD133 cells from tumors and not from contaminating normal stem cells, we determined the potential of this subpopulation to invade through Matrigel and survive in suspension culture (Fig. 2). Compared with the positive controls [a metastatic prostate cancer cell line (PC3M) and a metastatic breast cancer cell line (MCF7)], four of six cultures derived from prostate tumors were significantly more invasive than both PC3M and MCF7 \( (P < 0.005) \) and the remaining two of six tumor cultures were as invasive as the cancer cell line controls (Fig. 2A).

A further indication of tumor cell origin came from replating cells in semisolid medium (methylcellulose) and subsequently determining anchorage-independent survival. CD44\(^+/\alpha_2\beta_1^{hi}/CD133^+\) cells, from four separate tumors, showed enhanced survival relative to the unselected tumor cell population (median 3-fold higher) and even greater survival (median 8-fold higher) compared with the more differentiated CD44\(^+/\alpha_2\beta_1^{low}\) cells (Fig. 2B). On isolation of small cell populations, there is a finite possibility of cross-contamination between cultures. Indeed, this seems to have been a common occurrence in various tumor cell banks. To verify the identity of all cultures, a microsatellite analysis using a panel of five highly polymorphic loci was routinely done. Although this was unable to unequivocally confirm the tumor cell origin of the cultured cells, the microsatellite signatures matched cultures to the PBLs (and in some cases the original tissues) of the patient from which the culture was derived. All the long-term cultures were

**Table 2. CD133\(^+\) cells are maintained in long-term cultures of prostate cancer cells**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Days in culture</th>
<th>Percentage CD133 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10-28</td>
<td>28-31</td>
</tr>
<tr>
<td>228</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>434</td>
<td>0.4</td>
<td>1.2</td>
</tr>
<tr>
<td>484</td>
<td>1.5</td>
<td>2.5</td>
</tr>
<tr>
<td>563</td>
<td>1.2</td>
<td>0.6</td>
</tr>
<tr>
<td>605</td>
<td>0.3</td>
<td>0.9</td>
</tr>
</tbody>
</table>

NOTE: All values represent the percentage of CD133\(^+\) cells following MACS cell sorting.

**Figure 1.** CD44\(^+/\alpha_2\beta_1^{hi}/CD133^+\) cells have a high proliferative potential in vitro. **A,** primary colony-forming efficiency (CFE) of CD44\(^+/\alpha_2\beta_1^{hi}/CD133^+\), CD44\(^+/\alpha_2\beta_1^{hi}/CD133^+\), and CD44\(^+/\alpha_2\beta_1^{low}\) cells relative to unselected population (black columns). Secondary colony-forming efficiency relative to unselected population (white columns). Columns, mean of three experiments; bars, SD. **B,** the proliferative potential of CD44\(^+/\alpha_2\beta_1^{hi}/CD133^+\)–derived cells from prostate tumors. Two lymph node metastases (PE228, dashed line; PE605 (○), solid line), one poorly differentiated Gleason 9 (PE434 (●), solid line), two moderately differentiated Gleason 7 (PE484 (▲), dashed line; PE563 (△), solid line), and one benign prostate (PE352 (○), solid line).

**Figure 2.** Tumor cell origins of the in vitro cultures. **A,** in vitro invasion assay using cultures of one normal biopsy (benign prostate), two lymph node metastases (PE228, PE605), two poorly differentiated Gleason 8/9 (PE434, PE569), and two moderately differentiated Gleason 7 (PE484, PE563). Two positive controls (PC3M and MCF7) and one negative control (PNT1A). The percentage of cells migrating into Matrigel was measured in the presence of stromal conditioned medium as a chemoattractant in the medium below the Matrigel. Columns, mean of three experiments; bars, SD. **B,** survival of CD44\(^+/\alpha_2\beta_1^{hi}/CD133^+\) and CD44\(^+/\alpha_2\beta_1^{hi}/CD133^+\) cells in methylcellulose. Values shown are for survival relative to the unselected population \( (value = 1) \) from four independent patients. Surviving cells were counted after 28 days in complete methylcellulose medium. Note that similar populations of cells from benign prostate did not survive under these culture conditions. Mean (with SD) of four experiments.
of human origin and did not derive from the irradiated STO feeder cells. Two examples of this (for cultures P434 and P484) with three of five microsatellites are shown in Fig. 3. In two of six cultures (shown for tumor P434 with the microsatellite D8S264), evidence of microsatellite instability was observed but only in the tumor cultures and not in the patient's PBL DNA.

CD44+/α2β1hi/CD133+ cells differentiate to an androgen receptor–positive phenotype similar to prostate cancers in situ. To determine whether the tumor stem cell population was capable of differentiation to a secretory luminal phenotype, we applied conditions previously used to induce differentiation of normal prostate epithelium. As the prostate is an androgen-dependent organ, where the bulk population of tumor cells is responsive to the effects of androgen, cultures were treated with 1 nmol/L dihydrotestosterone in serum-containing medium and the resultant phenotypes were determined by flow cytometry. Immunocytochemical analysis of isolated CD133+ cells (purified either directly from disaggregated tumor samples or after expansion in culture) showed that this small population had a basal phenotype (Fig. 4A). CD133+ cells expressed the high-molecular-weight keratins 5 and 14, as well as keratin 19. Keratin 18 was weakly expressed and only in a minority of the population. c-MET, a marker of the transit-amplifying population (15), and the markers associated with the terminally differentiated cells in the prostate, androgen receptor, and prostatic acid phosphatase were not expressed. We also looked at prostate-specific stem cell antigen expression, which is a marker of intermediate prostate epithelial cells (16) and as expected it was not expressed within the CD133-selected population. Although the expanded tumor cultures were capable of some degree of differentiation under serum-free culture conditions, higher expression levels of the androgen receptor and prostatic acid phosphatase were only achieved after treating the cultures with serum and dihydrotestosterone (Fig. 4B). CD133 expression was also determined under differentiating conditions and was characteristically reduced [by fluorescence-activated cell sorting (FACS) analysis] but not eliminated after serum and dihydrotestosterone treatment in the two cultures examined (Table 3).

Discussion

We have previously established that the cell surface markers, CD44+/α2β1hi/CD133+, can be used to locate and isolate an epithelial
Table 3. CD133+ cell content of cultures decreases after differentiation treatment

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Undifferentiated</th>
<th>Differentiated</th>
</tr>
</thead>
<tbody>
<tr>
<td>434</td>
<td>3.63</td>
<td>1.33</td>
</tr>
<tr>
<td>228</td>
<td>0.53</td>
<td>0.06</td>
</tr>
</tbody>
</table>

stem cell population from normal human prostate (10, 11). The experiments we describe here constitute the first successful attempt to isolate and characterize the cancer stem cell population from human prostate tumors using identical markers. There are three lines of evidence to support that these cells are tumor stem cells: (a) they represent a small fraction of the total cells comprising the tumor, (b) they self-renew and proliferate, and (c) they differentiate to recapitulate the phenotype of the tumor from which they were derived.

Regardless of tumor grade, a small population of CD133-expressing cells could be isolated from a large series of prostate tumors, which included metastatic lesions. This proportion of stem cells is in contrast with a recent publication by Singh et al. (7), who reported that brain tumors, particularly high-grade medulloblastomas, contained two orders of magnitude higher numbers of CD133-expressing cells compared with prostate tumors. Nonetheless, the levels of CD133-expressing cells are more consistent with our previous findings with normal prostate epithelial stem cells (11), where the CD133+ cells constitute 1% of the basal population.

There are striking similarities between normal tissue homeostasis and tumorigenesis. Stem cells are functionally defined as self-renewing and multipotent, generating the mature cell types of a particular tissue through differentiation. In malignancies, such as leukemia (5) and breast (6), brain (8), and, more recently, lung (9) cancers, rare cells have been isolated with a remarkable potential for self-renewal. These cells were distinct from the bulk of the tumor in driving tumor growth and maintenance.

We found that only tumor-derived CD133+ cells were capable of self-renewal and extensive proliferation. In contrast, more differentiated phenotypes did not form secondary colonies and a comparison of the proliferative potential of CD133+ cells from benign prostate showed the capacity of tumor-derived CD133+ cells to proliferate extensively in vitro, which is similar to our previous findings with benign prostate (11). Thus, CD133 expression identifies an exclusive subpopulation of prostate tumor cells that are potentially immortal (having undergone >30 PD in culture to date) compared with similar populations of cells from nonmalignant prostate.

Although we acknowledge that the ultimate test of tumorigenicity is transplantation in vivo, we do provide compelling evidence that the CD133-derived cultures were tumorigenic as they were invasive in Matrigel (12) and survived in suspension culture at clonal densities. In our hands, most primary prostate tumor cells do not form large discrete colonies in methylcellulose (if plated at cloning densities), as found with mammary (17) and neural tumors (7).

The cultured cells were, in all cases used for biological characterization, identical by microsatellite analysis to the genotype of the patient from which they were derived. Although insufficient microsatellite markers were used to determine the extent of allelic imbalance, in two cases, microsatellite instability, which has been observed in a proportion of prostate tumor biopsies and associated with prostate cancer recurrence after therapy (18, 19), was detected in the cultured tumor stem cells. A fuller analysis of multiple loci and a detailed assessment of degrees of heritable genetic change by single nucleotide polymorphism analysis, with the Affymetrix 100k single nucleotide polymorphism chip using purified cell populations is currently under way. There is no certainty that the cancer stem cells will show the full extent of chromosomal damage found in previous analyses of prostate tissue DNA, as genetic heterogeneity in a large developing malignant tumor (sufficiently large for such analysis and often containing a significant stromal component) is likely to be great (14, 20).

The cellular origins of prostate cancer are still controversial. It has been suggested that prostate cancers arise from the terminally differentiated luminal cells (21) because the bulk population of tumor cells, in the most common form of prostate cancer, express luminal cell-specific markers (cytokeratins 8 and 18, androgen receptor, prostate-specific antigen, and prostatic acid phosphatase), but lack expression of basal cell markers, such as p63. Other studies suggest that the disease is derived from intermediate progenitors that have acquired the ability to self-renew (22). Xin et al. (23) recently reported that introduction of constitutively active AKT in Sca-1-enriched murine prostate epithelial cell results in the initiation of prostate tumorigenesis. Moreover, Sca-1-sorted cells, enriched for cells with prostate-regenerating activity, showed evidence of both basal and luminal lineage. Our data suggest that prostate tumors originate from basal cells expressing CD133 as this fraction exclusively had the ability to self-renew and differentiate into the mature cell types, which characterize the vast majority of prostate tumors.

By exploiting shared antigen expression between normal and cancer stem cells, we have been able to identify and isolate a population of cells that is highly enriched for a phenotype that is consistent with a cancer stem cell for prostate. With evidence of self-renewal, proliferation, and differentiation that recapitulates the original tumor phenotype, we have defined a class of prostate cancer stem cells that can be prospectively isolated from prostate tumors. These cells form a small subset of the population even after long-term culture. Therefore, we would predict that both tumor heterogeneity and the overall androgen-sensitive phenotype of human prostate cancers is the consequence of differentiation from an androgen receptor-negative stem cell population. Although further in vivo experiments are required to determine whether the tumor-initiating cells are comparable with the in vitro phenotype, this study provides evidence of the origin of prostate cancer from a CD133-enriched cell, that possesses stem cell properties and can serve as targets for the initiation of prostate tumorigenesis and metastasis.

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References

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