Electron Microscopic Analysis of Stem Cells in Human Prostate Cancer, Including Inverted Capsule Embedding Methods for Archival Sections and Falcon Films for Prostate Cancer Cell Lines

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Abstract. *Background/Aim:* Identification of prostatic stem cells in primary prostate tissue sections, organ cultures of prostate and cell lines requires a range of techniques that allows characterization of stem cells for their potential use in the treatment of patients. Isolated cells usually round-up and develop changes in shape, size and cellular characteristics. The aim of this study was to provide a range of methods for identifying prostatic stem cells and characterizing them with regard to ultrastructure, nuclear morphology, cytoplasmic organelles, and/or expression stem cell marker CD133. *Materials and Methods:* Prostate biopsy and prostatectomy specimens were used for studying prostatic stem cells and their known marker CD133 in tissue sections by light and/or electron microscopy. Inverted capsule embedding was used to study archival metastatic prostate in pelvic nodes and Du145 cell line in a monolayer culture. *Results:* Staining for CD133 positively identified stem cells that were found in benign prostatic hyperplasia, benign prostate, and prostate cancer cells. Paraffin embedded sections showed a single type of stem cells, whereas methylene blue-stained Epon sections showed both light and dark stem cells. Electron microscopy showed that both basal and stem cells were closely associated with the basement membrane (basal lamina). Stem cells had smooth plasma and nuclear membranes, a prominent nucleolus, small mitochondria, and few ribosomes. Du145 cells were separated

by intercellular spaces in monolayer culture. *Conclusion:* The inverted capsule embedding method allowed the study of metastasized prostate cancer in pelvic lymph nodes. Our approach enabled the assessment of stem cells in tissue sections by light and electron microscopy.

Stem cells are identified by staining for CD133 marker (1). Stem and basal cells are undifferentiated or poorly differentiated cells in the basal compartment whereas columnar/cuboidal and neuroendocrine cells are differentiated cells in the luminal compartment (2). Both stem and basal cells are closely associated with the basement membrane (1, 3-6). Cancer stem and normal stem cells often share molecular mechanisms and functional capabilities (7). Some basal cells have stem cell characteristics (8-11). Stem cells have an extensive capacity for self-renewal (12-14). The human prostate contains about 1% stem cells in benign (normal prostate), benign prostatic hyperplasia (BPH) and adenocarcinoma of the prostate (11, 15).

Identification of a small number of stem cells in tissue sections requires several approaches, including electron microscopy, immunostaining for the stem cell marker CD133 and/or other markers, such as alpha-1 B₂-integrin (2, 10, 15-17). CD133 has been used to identify cancer stem cells in prostate and many other solid cancers (17). Any single approach has certain limitations, therefore, multiple approaches are required, especially for isolated stem cells in culture and tissue sections.

Human prostate cancer (PC) is a genetically, morphologically and pathologically heterogeneous tumor, and PC complexity is further increased by its regulation by a variety of steroid hormones (namely, testosterone, estrogen, progesterone) (2). Stem cells are independent of steroid hormones (18). Smith *et al.* have concluded that normal prostate and cancer stem cells have similar characteristics (7). Various mutations occur in stem cells imparting their self-renewal properties (13, 18, 19). Basal and stem cells in the

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basal compartment do not degenerate following androgen deprivation therapy (ADT) in human prostate or after castration in mice (2, 6). Stem cells are immune to androgen-based therapies (20, 21) and other therapies (17). In castrated mice, testosterone treatment triggers proliferation of stem cells followed by their differentiation into columnar/cuboidal cells (22). In contrast, luminal (columnar/cuboidal) cell degenerate following ADT in human prostate and some stem cells differentiate in human prostate following testosterone treatment (2, 6). The presence of androgen and estrogen receptors in some untreated and DES (diethylstilbestrol)-treated stem cells indicates that they are destined to differentiate into secretory cells. Some stem cells do not develop receptors and remain as stem cells (2, 23). We hypothesized that characterization of stem cells by ultrastructure, nuclear morphology, cytoplasmic organelles, and stem cell marker CD133, including utilization of inverted capsule embedding methods and growing cell lines on Falcon films may provide a range of criteria to identify stem cells in tissue sections, as well as when cells are isolated from prostate cancer sections.

Materials and Methods

Inverted capsule embedding method. We modified the inverted capsule embedding method used by others (23-25). Briefly, we utilized archival prostate tissue sections that were used in diagnosis of cancer. This involved deparaffinization of sections in xylene and processing them via graded ethanol to water and re-fixation in a combination of buffered 2% paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.3 for 2 h, as previously described (26, 27). Thick sections were washed in phosphate buffered saline (PBS) (4 changes), dehydrated in graded ethanol and absolute alcohol (26). Gelatin capsules were filled to the brim with Epon 812 and capsules were inverted on to the slides containing tissue sections and allowed to polymerize for 2 days at 60°C. Slides were air cooled to room temperature and capsules were lifted from the slide that contained embedded areas (Figure 1b). Capsules containing appropriate areas were sliced to create thick sections (1-2 (μm) for light microscopy and thin sections (about 400-500 angstrom) for electron microscopy. Prostate cancer and metastasized PC to the pelvic lymph node were fixed using the above approach. Paraffin sections were stained with-hematoxylin for light microscopy (Figure 1c). Thin sections were processed for electron microscopy as reported previously (26, 27).

Falcon film method. Certain prostate cell lines (such as LNCaP and Du-145) have played an important role in establishing tumor models for a variety of experiments. For example, Ohtsuki *et al.* have grown cells in Beem capsule for electron microscopy (28). Briefly, Du-145 prostate cells were grown as monolayer on Falcon 3006 film-lined dish (Becton Dickinson Labware Oxnard, CA, USA), fixed in 3% buffered glutaraldehyde, washed in PBS, post-fixed with 1 to 2% buffered osmium-tetroxide for 1-2 h, washed again and embedded in Epon. Thick and thin sections were made using Reichert -Jung microtome (C. Reichert, Wien, Austria) for transmission electron microscopy. Thin sections were stained with lead citrate and uranyl acetate (Polysciences, Inc, Warrington, PA, USA) (26, 27).

Former Veterans Affairs Medical Center (VAMC) urology surgeon, Dr. Clyde E. Blackard and his associates, selected patients for biopsy and/or radical prostatectomy. Patients were not treated with any hormone therapy or chemotherapy prior to biopsy and prostatectomy. Prostate specimens were submitted to the Pathology Service of Minneapolis VAMC. Specimens not used in diagnosis of PC were collected for research between 1972 and 1975, embedded in Epon and stored. Prostate samples were obtained following the approval of the institutional review board (IRB) guidelines in place at the Department of Veterans Affairs (VA) and the University of Minnesota. Prostatectomy and/or biopsy tissue samples were fixed for two h using the combination of 2% paraformaldehyde and/or 3% glutaraldehyde in 0.1M phosphate buffer at pH 7.3 (26, 27). Briefly, prostate pieces were washed in buffer and post-fixed in 1% to 2% buffered osmium-tetroxide, washed again, dehydrated in graded ethanol, and embedded in Epon 812, as previously described. Areas of interest were sliced for thick and thin sections using a Reichert-Jung microtome. Thin sections (about 400 -500 angstrom) were mounted on copper grids, stained with a combination of lead citrate and uranyl acetate and examined with RCA EMU 3 or 4 electron microscopes, as detailed (26, 27, 29). Sections were graded by Drs. Donald F. Gleason and Nancy A. Staley, former staff pathologists at the Minneapolis VAMC. In addition, paraffin sections stained with or without hematoxylin and eosin were obtained for further study. A total of 25 untreated cases included only 4 BPH. They were examined by light and electron microscopy. The age of untreated patients ranged from 58 to 79 years and the mean±standard error of the mean was 70.54±3.60. Patients had PC with pathological grades III and IV tumors which are comparable to Gleason histological scores 6 to 10 (30). Clinical stages were B, C and D (31). Benign prostatic hyperplasia and cancer pieces were used for localization of CD133 as we have previously described (2).

Results

Micrograph shows cellular details and nuclear morphology of Du-145 cells and the intercellular spaces (Figure 1a). Areas of sections lifted with the inverted capsule embedding method and the areas not embedded in the capsules are shown in Figure 1b. Metastasized prostate cancer cells are illustrated in the pelvic lymph nodes, which also show numerous lymphatic cells (Figure 1c). CD133 positive stem cells were identified in paraffin sections of benign prostatic hyperplasia and benign prostate (Figure 2a). Paraffin embedded sections showed a single type of stem cells, whereas methylene blue stained Epon sections showed light and dark stained stem cells, as described earlier (32) (Figure 2b and c).

Dark cells stain for basic protein whereas light cells do not (2, 32). Light stained stem cells were near the basement membrane and had round to oval nucleus with nucleolus (Figure 2c). Dark stained stem cells were also near the basement membrane (Figure 2c). Electron microscopy showed that the basal cells were closely associated with the basement membrane (basal lamina) in benign prostate, BPH and PC (Figure 2c). Electron micrograph illustrates two electron lucent stem cells (or light stem cells) (Figure 2d).

Figure 1. *a. Electron micrograph of Du145 prostate cells shows nuclei, cytoplasmic organelles and interstitial spaces between cells. A dividing cell is showing aggregates of chromosomes. Bar shows magnification. b. Metastatic prostate in lymph node was embedded in Epon using inverted gelatin capsule method. Areas not embedded in capsules remain on the slide and those embedded in the capsules are removed. Bar shows magnification. c. Thick section illustrating metastatic prostatic glands among the nodal lymphocytes. Prostatic gland shows stem cell (arrows) and acinar lumen is shown by L. Bar shows magnification.*

Stem cell cytoplasm has small mitochondria and few ribosomes. Golgi complex or secretory vacuoles and granules are found in differentiated cells (Figure 2d). Nuclei have smooth nuclear membranes in basal and stem cells, but columnar cells have either smooth and distorted nucleus and

nuclear membrane (or nuclear plasticity) (Figure 2d). Micrographs also showed round to oval nucleus and nucleolus and relatively sparse cytoplasmic organelles in stem cells (Figure 3a). Columnar/cuboidal cells have numerous secretory granules, mitochondria and ribosomes.

Figure 2. a. Stem cells in benign prostatic hyperplasia are shown by CD133 immunostaining. Glandular lumen and stroma do not show labelling indicating specificity of staining. Section was not counterstained (Prostate 473). Bar shows magnification. b. Localization of CD133 immunostaining in stem cells in PC. (Case #391). Bar shows magnification. c. Methylene blue stained Epon section illustrates light stained stem cell (arrow) and dark stained stem cell (arrow heads). Label 'c' is in prostatic stroma. (Case #40). Bar shows magnification. d. Electron micrograph illustrates two electron lucent stem cells (or light stem cells) also shown in figure (1c). Nuclei have smooth nuclear membranes, cytoplasmic organelles are sparse, and the cell is poorly differentiated. These cells are closely associated with each other and the adjacent secretory cells. Columnar cell nucleus is distorted (or nuclear plasticity) (Case #40). Bar shows magnification.

In methylene blue-stained thick sections, stem cell nucleus was electron lucent or electron dense, which was comparable to light and dark cells in untreated cases reported previously (2). CD133 immunogold gold particles were identified in both light and dark stem cells. Thus, the

basal compartment of the prostate contains basal and stem cells, which are undifferentiated cell types (Figure 3a). Columnar/cuboidal cells reached both the basement membrane and acinar lumen and had elongated nucleus along the baso-lateral axis (Figure 3a, b). Another stem cell

Figure 3. *a*. A low-power electron micrograph illustrates many acinar cells and acinar lumen (L). A basal cell with dark stained nucleus is shown by an arrow head and adjacent two stem cells are shown by two arrow heads. Acinar cells show secretory granules and their nuclei. Stem cells have smooth nuclear membrane, and prominent nucleoli and small mitochondria. Cytoplasmic organelles in stem cells are sparse and poorly differentiated and are comparable to figure 2d. (Case #110). Bar shows magnification. *b*. Another stem cell with nucleus illustrating aggregates of chromosomes within a smooth nuclear membrane and relatively sparse cytoplasmic organelles. The stem cell is closely associated with secretory cells showing secretory granules and vacuoles and a part of secretory cell nucleus. The labelling (b) is in the stroma. (Case #11). Bar shows magnification. *c*. Another stem cell illustrating aggregates of chromosomes within a smooth nuclear membrane and relatively sparse cytoplasmic organelles. This stem cell is closely associated with two other stem cells. Each stem cell has smooth nuclear membrane euchromatin and prominent nucleolus. Each stem cell is undifferentiated and has small mitochondria and ribosomes. (Case #103). Bar shows magnification.

shows aggregates of chromosomes within a smooth nuclear membrane and has relatively sparse cytoplasmic organelles. This stem cell is closely associated with two other stem cells (Figure 3c). Each stem cell is undifferentiated and has small mitochondria and ribosomes.

Discussion

Basal and stem cells are closely associated with each other and with acinar cells by desmosomes of tight junctions. In the past, lighter and darker stained basal cells had been observed by Kirchheim and Bacon (32) and subsequently by our group (27). We found that light and dark stained stem cells existed in untreated and DES-treated cases (27). Light stained stem cells and their progeny were androgen-sensitive, whereas dark stained stem cells were androgen-independent. Recently, we have shown that light stained cells have androgen receptors and dark stained cells have estrogen receptors (6). In the present study, basal and stem cells were readily identified by electron microscopy with and without staining for the stem cell marker, CD133 (2). In the past, studies have mostly focused on the cytological features of differentiated prostatic cells (32, 33). Electron microscopy allowed the assessment of stem cells by nuclear shape, distribution of heterochromatin and chromatin, cytoplasmic organelles, namely, small mitochondria and few ribosomes. Morphological features distinguish stem cells in tissue sections and metastatic prostate in the pelvic nodes. Staining for CD133 provided an additional method of identification of stem cells (12, 16, 17, 34).

Electron microscopy allowed the assessment of cellular details and nuclear morphology of Du145 cells and intercellular spaces between the cells. Collins *et al.* have used collagenase digestion of prostate tissue and Becton-Dickinson FACS-Scan method to study isolated stem cells (15, 16). Lang *et al.* have grown spheroids of prostate cell lines (PC-3) in Matrigel and analyzed their morphological features by light and electron microscopy (35). They have found differentiated luminal epithelial cells. The inverted capsule method allowed the analysis of metastatic prostate in pelvic lymph nodes. Metastatic prostate samples are difficult to obtain, but they can be readily processed for the inverted capsule embedding methods. This has enabled the assessment of interactions between metastatic cells and lymphatic cells in pelvic lymph nodes (36). In addition, the inverted capsule approach can be helpful in the study of metastases in archival solid organ cancers, namely, breast, colon, lung and brain cancers.

Conflicts of Interest

The Author has no conflict of interest to report regarding the publication of this article. The opinion expressed in this article is that of the author and not of the U.S. Government, Department of Veterans Affairs or the University of Minnesota.

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