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PROGENITOR CELLS ARE RESPONSIBLE FOR FORMATION OF HUMAN PROSTATE EPITHELIUM PRIMARY CULTURES

*T. Drewa[1, 2, *], Z. Wolski[1], L. Pokrywka[1], R. Debski[3], J. Styczynski[3]*

[1]*Department of Urology, Nicolaus Copernicus University (NCU), Bydgoszcz, Poland*
[2]*Department of Tissue Engineering, Chair of Medical Biology, NCU Bydgoszcz, Poland*
[3]*Department of Pediatric Hematology and Oncology, NCU, Bydgoszcz, Poland*

Aim: **To analyze cell viability and morphology of primary cell cultures from CD133 immunolabeled and sorted cells from epithelium of patients suffering from benign prostate hyperplasia (BPH).** ***Methods:*** **Cells obtained from 5 patients were divided in two fractions. First fraction ($CD133^+/CD133^-$) was cultivated in DMEM with 10% FBS. Second fraction was mixed with CD133 microbeads and immunomagnetically divided into $CD133^+$ and $CD133^-$ fractions. These cells were cultivated and followed-up for 2 weeks. Cells were stained for Annexin V FITC/propidium iodide.** ***Results:*** **Seventy $CD133^+/CD133^-$ cultures, thirty-one of $CD133^+$ and thirty-one of $CD133^-$ cells were established. There were 5-fold and 3-fold increase of $CD133^+/CD133^-$ and $CD133^+$ cell number after 2 weeks, respectively. $CD133^+/CD133^-$ and $CD133^+$ monolayers displayed epithelial-like morphology and cytokeratine expression. $CD133^-$ cultures collapsed. Cell viability within $CD133^+$ and $CD133^-$ populations was $90.1 \pm 6.3\%$ and $24.3 \pm 6.2\%$, respectively. Apoptotic index was $9.0 \pm 6.1\%$ and $28.5 \pm 23.8\%$ within $CD133^+$ and $CD133^-$ cultures, respectively.** ***Conclusions:*** **CD133 separated human primary epithelial cell cultures displayed differences in morphology, viability and apoptosis occurrence. Immunomagnetic sorting can be recommended in each *in vitro* experiments with primary cell cultures in order to provide more objective results.**

Key Words: **primary epithelial cell culture, human prostate, benign prostate hyperplasia, cell viability, apoptosis, flow cytometry.**

We often observe the discrepancy between the results obtained from *in vitro* and *in vivo* studies. Many substances were proved to act perfectly *in vitro* studies, but their *in vivo* action was usually unsuccessful. One can argue that these divergences are connected with *in vitro* models, which are based on the immortalized and commercially available cell lines. Striking differences between results establishing on cell culture systems *vs* animal models are even observed if the primary cell cultures were used.

Normal prostate epithelial stem cells were identified and found to have a basal cell phenotype together with the expression of CD133 antigen [1]. The establishment of the isolation method of $CD133^+$ single-stem cell suspension from adult prostatic tissue was reported [2]. It was proven on rat model that progenitor cells were responsible for establishing the primary prostate epithelial cultures [3].

In this paper we analyzed the short-term primary culture of CD133 immunolabeled and sorted cells from epithelium of patients suffering from benign prostate hyperplasia (BPH). We will try to find the differences of cell viability and morphology between CD133 sorted cells during the formation of monolayers.

METHODS

Prostate epithelium cell culture $CD133^+/CD133^-$. Prostate specimens were obtained from 5 patients suffering from BPH treated by an open prostatectomy. Specimens of ca. 2 cm^3 were resected from central zone. Specimens were chopped into small pieces of 0.5 x 0.5 x 0.5mm in order to facilitate enzyme access to epithelial cells. Tissue was digested 6 h with 0.5% colagenase (Sigma, Germany) in a stirrer. Cell suspensions obtained after digestion were passed through 30 µm mesh (Sigma) to remove cell clumps. Digested cells were counted using trypan blue exclusion assay. Homogenous cell suspensions were divided into two equal fractions. Cells from the first part were seeded on 25 cm^2 T-flasks. These cultures were considered as $CD133^+/CD133^-$ and cultivated in DMEM with 10% FBS and supplements in atmosphere of 5% CO_2 with suitable humidity. Due to extensive culture growth medium was changed every two days. The first medium exchange was partially, i. e. only half of medium was removed and then in the next procedures always the whole volume of medium was changed. The epithelial nature was confirmed using anti-cytokeratine antibody (Pancytokeratine, clone MMF118, Dako, Denmark). Procedures were approved by Local Bioethical Committee.

$CD133^+$ cells magnetic isolation, $CD133^+$ and $CD133^-$ culture. Phosphate buffered saline pH 7.2 supplemented with 0.5% bovine serum albumin (Gibco) and 2 mM EDTA (Sigma) was prepared. Buffer was degassed in the ultrasound bath and kept in the refrigerator. Cells from the second fraction were suspended in 300 µl degassed buffer. 100 µl FcR Blocking Reagent was added to inhibit unspecific binding of antibodies to non-target cells. Cold solutions were used to avoid capping of antibodies on the cell surface during labeling and avoid nonspecific cell labeling. Cells were labeled by adding 100 µl CD133 MACS MicroBeads (Miltenyi Biotec, Bergish Gladbach, Germany). Suspensions were mixed and incubated for 30 min at 4 °C.

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*Correspondence: Fax: +48525854045
E-mail: tomaszdrewa@wp.pl

Abbreviations used: ABCG2 – ATP binding cassette G2 protein; AR – androgen receptor; BPH – benign prostate hyperplasia; DMEM – Doulbecco's minimal essential medium; FITC – Fluoresceine isothiocyanate; PI – propidium iodide; PSA – prostatic specific antigen.

Cells were washed by adding 10 x the volume of buffer and centrifuged at 300 g for 10 min. Supernatant was removed and pellet was re-suspended in 500 µl buffer. MS Columns (Miltenyi Biotec) were used for magnetic separation in SuperMACS II device (Miltenyi Biotec). After pre-wash of the column with 500 µl of buffer, cell suspension was applied onto the column to allow the non-labeled cells to pass through. Then the column was washed with 2000 µl of buffer. Obtained cell suspension was collected in T-flask and cultivated in conditions described above. This culture was considered as $CD133^-$. Then the column was removed from magnetic field and placed on collection tube. Magnetically labeled cells were flushed out by using 1000 µl of buffer. $CD133^+$ cell suspension obtained in this way was cultivated. In these cultures medium was changed for the first time after 4 days and then every 3 days till the end of experiment. Medium in $CD133^-$ cultures was changed only 3 times during observation period. The first medium changing was partially, in order to protect unattached living cells. After two weeks of culture cells growing in monolayers were counted again using trypan blue exclusion assay.

Flow cytometry study. Living, apoptotic and necrotic cells were counted after 2 weeks of culture. In order to detect living and apoptotic cells by flow cytometry Annexin V conjugated with fluoresceine isothiocyanate (Annexin V–FITC) and propidium iodide (PI) staining kit (Immunotech, Beckman-Coulter, US) was used. Living cells were negative for Annexin V-FITC and PI. Early apoptotic cells were detected after binding with Annexin V-FITC. Secondary necrotic cells stained positively both for Annexin V-FITC and PI. It is presumed that secondary necrotic cells died in apoptotic process. Necrotic cells stained with PI. For flow cytometry study all cells from both monolayers and suspensions were counted using trypan blue exclusion assay. Then 10^5 centrifuged cells were suspended in 0.5 ml of binding buffer containing 0.05 ml Annexin V-FITC and 0.05 ml PI. The analysis was performed using the FC 500 flow cytometer (Beckman-Coulter, Miami Fl, US) with CPX Software. All experiments were performed at least in triplicate.

RESULTS

Material for 132 separate cultures was isolated. 70 cultures were $CD133^+/CD133^-$, 31 were $CD133^+$ and 31 were $CD133^-$. Each $CD133^+/CD133^-$ culture had approximately $4.0 \pm 0.1 \times 10^5$ isolated cells. Each $CD133^+$ labeled fraction had the mean number of $3.0 \pm 0.1 \times 10^4$ cells. Each fraction of non-labeled cells had $3.5 \pm 0.1 \times 10^5$ and was considered as $CD133^-$ culture. High proliferation rate within $CD133^+/CD133^-$ cultures was observed (Fig. 1, *a–e*). There were approximately $2.0 \pm 0.5 \times 10^6$ cells in each $CD133^+/CD133^-$ culture after 2 weeks of follow up (Fig. 2). $CD133^+$ cultures showed lower proliferation rate during the same observation period (Fig.1, *f–k*). The cell number of primary $CD133^+$ fraction was triplicated up to $9.0 \pm 0.4 \times 10^4$ cells in an each culture (Fig. 2). $CD133^+/CD133^-$ and $CD133^+$ monolayers displayed epithelial-like morphology (Fig. 1, *e* and *k*). $CD133^+/CD133^-$ colonies grew from groups of highly proliferated cells, which formed "nests", immediately after isolation (Fig. 1, *a–c*). The morphology and proliferation of $CD133^+/CD133^-$ cultures resembled keratynocyes growth obtained after digestion of epidermal sheets [4, 5]. $CD133^+$ colony has grown usually from single round-shape cell, but in the end of experiment the epithelial like monolayer was good visible (Fig. 1, f–h). It was possible to perform the first passage of $CD133^+/CD133^-$ and $CD133^+$ cultures after 2 weeks of culture. There was sufficient cell number in flasks covered with cells growing in monolayers. No culture of anchorage cells from $CD133^-$ populations were obtained during the 2 weeks of observation (Fig. 1, *m–r*). The primary $CD133^-$ cultures collapsed within this period. The suspension containing only a few $CD133^-$ cells was observed (Fig. 1, *r*).

$CD133^+$ populations showed the highest viability, which reached $90.1 \pm 6.3\%$ and the lowest percent of the apoptotic cells ($9.0 \pm 6.1\%$). Moderate viability was found when $CD133^+/CD133^-$ populations were examined. Among $CD133^-$ cultures percentage of living and apoptotic cells was $24.3 \pm 6.2\%$ and $28.5 \pm 23.8\%$, respectively (Fig. 3).

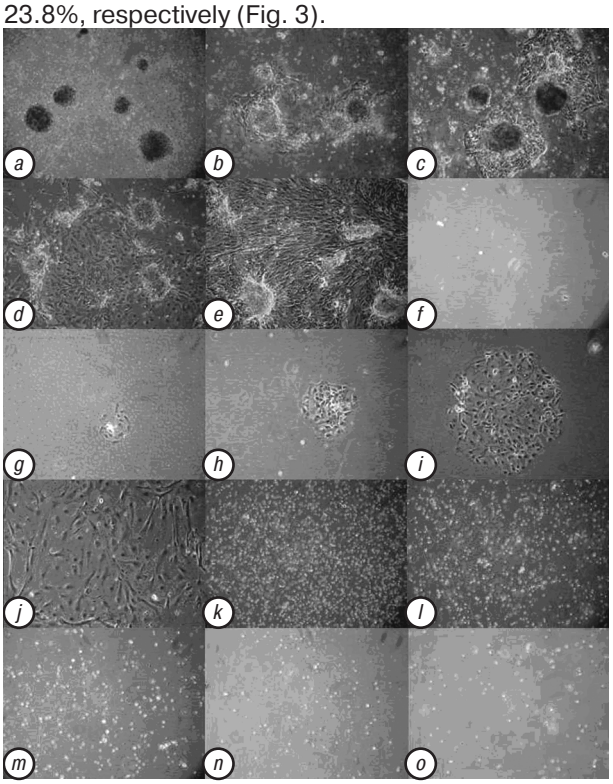

Fig. 1. $CD133^+/CD133^-$ cells in 0, 1, 2 and 7 days after enzymatic digestion of the human prostate (*a–d)*. (*e)* The confluent $CD133^+/CD133^-$ epithelial-like culture can be observed in 2 weeks. The epithelial-like monolayers of $CD133^+/CD133^-$ cells are growing from clusters of characteristic nests (*a*). (*f–j)* Present $CD133^+$ cells within the 0, 1, 2 and 7 days after isolation. (*k)* $CD133^+$ cells form monolayers similar to $CD133^+/CD133^-$ epithelial prostate culture obtained from simple tissue digestion after 2 weeks. (*m–p*) Present $CD133^+$ cells within the 0, 1, 2 and 7 days after isolation. (*r)* $CD133^-$ cultures failed to grow in two weeks upon cultivation (inverted microscope, magnification 100 x)

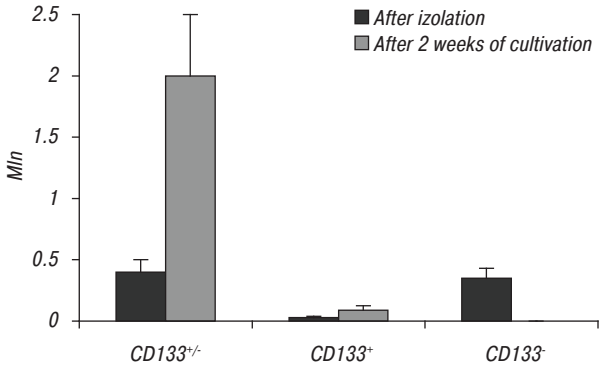

Fig. 2. The mean cell numbers in monolayers (only attached cells were counted) within primary cultures of the CD133$^+$/CD133$^-$, CD133$^+$ and CD133$^-$. Cells were counted using trypan blue exclusion test just after isolation, and then in two weeks upon cultivation. CD133$^-$ cells did not form monolayer in 2 weeks upon cultivation

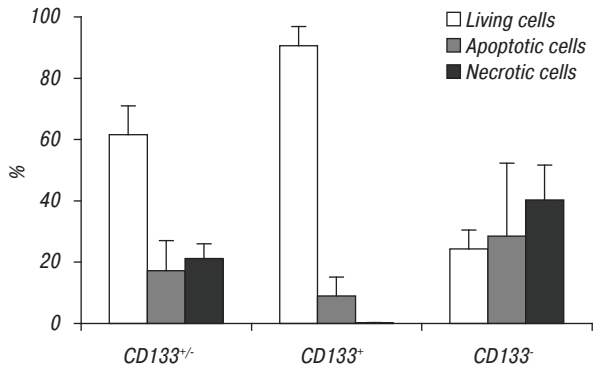

Fig. 3. The percentage of living, apoptotic and necrotic cells within the each primary culture of CD133$^+$/CD133$^-$, CD133$^+$ and CD133$^-$ populations in 2 weeks upon cultivation

DISCUSSION

Prostate epithelial stem cells are self-renewing cells capable of differentiation into prostate epithelium. Rodent models and immortalized or genetically modified cell lines are frequently used, but with limited utility for studying human prostate development. Adult stem cells (ASC) and transit-amplifying cells (TAC) have been observed in many tissues including prostate [6]. Epithelial cells probably share common stem cell markers [7]. Analysis of stage-specific differentiation markers has revealed that prostate stem cells were characterized by following markers: CD133$^+$/ABCG2$^+$/α(2)β(1)(Hi). These cells were able to self-renew and generate two distinct cell lineages. CD133$^-$/ABCG2$^-$/α(2)β(1)(Hi)/p63$^+$/PSCA$^-$/AR$^-$/PSA$^-$ were identified as transit-amplifying cells, while CD133$^-$/ABCG2/α(2)β(1)(Lo)/p63$^-$/PSCA$^+$/AR$^-$/PSA$^-$ were intermediate quiescent cells. Luminal-secretory cells were described as CD133$^-$/ABCG2$^-$/α(2)β(1)(Lo)/p63$^-$/PSCA$^-$/AR$^+$/PSA$^+$ [8]. It was shown that isolation of stem cells from normal and malignat prostate may now be possible [1, 3, 7, 9, 10].

There are two populations of cells capable of regenerating prostatic tissue. The first population (with considerable growth potential) resides in the proximal region of ducts and in the urethra, and the survival of these cells does not require the presence of androgens. The second population (with more limited growth potential) is found in the remaining ductal regions and requires androgens for survival [11]. We proved that CD133$^-$ cells had no potential for *in vitro* proliferation, even in the serum conditioned medium. Serum was used in this experiment due to obtain high cell viability and to facilitate anchorage growth in culture. We think that serum influence on differentiation during this short experiment was minimal. It can be speculated that these cells (CD133$^-$) probably have no ability to regenerate the prostate ducts *in vivo,* but it should be checked on animal model.

Richardson et al. [2] found that 1% of human prostate basal cells express the cell surface marker CD133. These cells (CD133$^+$) expressed α(2)β(1)(Hi) previously shown to be a marker of stem cells in prostate epithelia. We observed in our study that after isolation even 7% of cells were in CD133$^+$ fraction. This can be partialy explained by the fact, that cultures were established from BPH patients. There is a suspicion that BPH development originates from disturbances of stem cell proliferation. In proliferative diseases homeostasis of cell number is disrupted. Increased ability of stem cells to mitosis can lead deregulation of epithelial lining [7, 9, 12]. The second reason is that samples for culture were taken from central part of the prostate, including proximal parts of prostatic ducts. The proximal region has been suggested as the stem cell niche [12]. We obtained samples from the central zone located close to the urethra, where the possibility to isolate more CD133$^+$ cells seemed to be higher.

It was interesting that co-cultures of CD133$^+$ and CD133$^-$ populations have the higher proliferation rate, 5-fold increase of cell number during the two weeks (Fig. 2). On the second hand these co-cultures where characterized by moderate cell viability (Fig. 3). We think that the source of high proliferation rate within these cultures comes from cell-to-cell interactions and cannot be simply characterized by cell viability or apoptotic assays. The behavior of CD133$^+$ cells in culture resembled progenitors properties. The viability of these cells after detachment reached 100% in some cases, but proliferation rate was lower when comparing to CD133$^+$/CD133$^-$ co-cultures. CD133$^-$ cultures are probably composed of differentiated cells without clonogenic potential. These cells were not able to anchor and form monolayer. Some of them were still alive, but the most of them enter apoptotic pathway. The apoptotic induction amnong these cells were probably due to lack of anchorage growth [13, 14]. The behavior of separated cells is converged with the description of naturally occurring cell senescence process within the prostate epithelium [15]. We think that in experiment performed on primary cell culture at least three parts of growing tissue i. e.; progenitors, differentiating and committed to die cells have to be separately analyzed. There is an increasing evidence that stem cells are responsible for both cancer development and cancer treatment failure [5, 12]. In this light, it is very important to plan the *in vitro* experiment on benign or cancer cells with the use of these separated populations.

These suggestions are related also to established and commercially available cell lines [16]. Experiments performed on cell lines never considered progenitors as a part of tested cell population. May by this is the reason of discrepancy between the results obtained from *in vitro* and *in vivo* experiments [17]. We postulate that cell sorting would come closer results from *in vitro* experiments to *in vivo* conditions. If one analyses the cell viability its clear that $CD133^+/CD133^-$ viability is "a mean" calculated from $CD133^+$ and $CD133^-$ viability (see Fig. 3). The similar can be observed when apoptotic index was analyzed. The number of apoptotic cells within $CD133^+/CD133^-$ population can be estimated as "a mean" calculated from $CD133^+$ and $CD133^-$ alone, but this simplistic model cannot answer the questions arising from experiments where new substances or drugs are tested *in vitro*. It is, of course difficult or even impossible to predict results of such performed experiments, but it can be speculated that $CD133^+$ and $CD133^-$ cells would react in different ways. Immunomagnetic labeling is a simple method for cell sorting, which can be applied in every *in vitro* experiment to provide more objective results.

CONCLUSIONS

Epithelial progenitors are necessary to establish human primary prostate epithelial cultures *in vitro*. Interactions between stem and differentiated cells are crucial for growth and proliferation within prostate epithelial monolayers *in vitro*. Separated human primary epithelial cells fractions displayed differences in morphology, viability and apoptosis occurrence. Immunomagnetic labeling is a simple method for cell sorting, which can be applied in every *in vitro* experiment to improve results.

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РОЛЬ КЛЕТОК-ПРЕДШЕСТВЕННИКОВ В ФОРМИРОВАНИИ ПЕРВИЧНЫХ ЭПИТЕЛИАЛЬНЫХ КЛЕТОЧНЫХ ЛИНИЙ РАКА ПРЕДСТАТЕЛЬНОЙ ЖЕЛЕЗЫ

Цель: **оценить жизнеспособность и морфологию клеток первичных клеточных культур, полученных из меченных по CD133 и полученных с помощью клеточной сортировки клеток эпителия пациентов с доброкачественной гиперплазией предстательной железы (BPH).** ***Методы:*** **клетки, полученные от 5 пациентов, были разделены на 2 фракции. Первую фракцию ($CD133^+/CD133^-$) выращивали в DMEM с 10% FBS. Вторую фракцию смешали с CD133 магнитными гранулами и с помощью магнита разделили клетки на $CD133^+$- и $CD133^-$-фракции. Далее клетки культивировали в течении 2 нед. Клетки окрашивали аннексином V FITC/пропидий йодидом.** ***Результаты:*** **получено 70 $CD133^+/CD133^-$-культур клеток, 31 $CD133^+$ и 31 $CD133^-$. Через 2 нед культивирования отмечали 5-кратное и 3-кратное увеличение количества $CD133^+/CD133^-$ и $CD133^+$ клеток соответственно. $CD133^+/CD133^-$- и $CD133^+$-клетки росли в монослое и имели морфологию эпителиальных клеток, экспрессировали цитокератин. $CD133^-$-клетки не выжили. Выживаемость клеток в популяциях $CD133^+$ и $CD133^-$ была 90,1 ± 6,3% и 24,3 ± 6,2% соответственно. Показатель апоптического индекса для культур $CD133^+$ и $CD133^-$ был 9,0 ± 6,1% и 28,5 ± 23,8% соответственно.** ***Выводы:*** **показаны различия в морфологии, выживаемости клеток и частоте апоптоза для эпителиальных клеток, разделенных в зависимости от экспрессии CD133. Сортировка клеток с помощью иммуномагнитного разделения рекомендована для каждого *in vitro* эксперимента с использованием первичных клеточных культур для получения более объективных результатов.**

Ключевые слова: **первичные эпителиальные клеточные линии, предстательная железа, доброкачественная гиперплазия предстательной железы, выживаемость, апоптоз, проточная цитометрия.**