Original Research

Rapid adherence to collagen IV enriches for tumour initiating cells in oral cancer

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Received 22 May 2014; received in revised form 12 August 2014; accepted 15 September 2014
Available online 10 October 2014

Abstract  

\textbf{Background:} Although several approaches for identification and isolation of carcinoma cells with tumour initiating properties have been established, enrichment of a population of pure and viable tumour-initiating cells (TICs) is still problematic. This study investigated possibilities to isolate a population of cancer cells with tumour initiating properties based on their adherence properties, rather than expression of defined markers or clonogenicity.

\textbf{Methods:} Several human cell lines derived from oral dysplasia and oral squamous cell carcinoma (OSCC), as well as primary cells derived from patients with OSCC were allowed to adhere to collagen IV-coated dishes sequentially. Rapid adherent cells (RAC), middle adherent cells (MAC) and late adherent cells (LAC) were then harvested and further investigated for their morphology, stem cell-like properties and molecular profile while grown in vitro and tongue xenotransplantation in NOD-SCID mice at serial dilutions.

\textbf{Results:} RAC showed significantly higher colony forming efficiency ($p < 0.05$), sphere forming ability, greater migration ability ($p < 0.05$), exhibited longer G2 phase and displayed higher expression of integrin $\beta_1$ and other stem-cell related molecules as compared to MAC and LAC. RAC induced tongue tumours in NOD-SCID mice with the highest incidence. These tumours were also bigger and metastasised more frequently in loco-regional lymph nodes than MAC and LAC.
1. Introduction

Oral squamous cell carcinoma (OSCC) has poor prognosis due to therapy-resistant loco-regional recurrences and distant metastases [1]. The cancer stem cell theory, although controversial, seems to explain well the behaviour of this disease, and the existence of a subpopulation of cells with tumour initiating properties and increased self-renewal has been shown in OSCC [2]. Understanding the biology of tumour initiating cells (TICs) is thus essential for better understanding and more specific targeting of OSCC. For the same reason, robust methods for isolation of TICs are in demand. Numerous strategies for enrichment of TICs have been previously described [2–5]. However, isolating a population of pure and viable putative TICs is still problematic. Fluorescence-activated cell sorting (FACS) is the most common technique to isolate TICs based on specific surface markers, and CD44 has been repeatedly reported to identify human oral cancer stem cells [2,4]. Nevertheless, there is no single marker that can be used for isolation of TICs for any type of cancer, including OSCC, because of tumour heterogeneity and lack of reproducibility [5]. Moreover, the FACS technique requires high-cost, high-speed sorters and high-quality antibodies. Sorting the side population (SP) on the basis of the ability to efflux the fluorescent DNA-binding dye Hoechst 33342, or sorting of cells based on the ALDH1 enzymatic activity has also been used for isolation of TICs, but sometimes only few cells are acquired by these methods, and thus they are hardly of any use for further investigations [5]. In addition, the method of sorting SP cells cannot be applied broadly since not all cell lines contain SP cells [6]. Recent studies that explored more functional, non-invasive alternative methods to FACS analysis, demonstrated that non-adherent sphere formation [3], in vivo serial tumour xenotransplantation [7], as well as chemotherapeutic drug resistance [7,8] could be used to enrich a subpopulation with TICs. However, these methods require a long time to yield TICs, and not all cell types and cell lines are able, for example, to grow as spheres. In this study we have assessed the potential of a functional approach for TIC isolation in OSCC, a method previously used for normal epithelium [9]. Although severely disturbed, OSCC has an organisation resembling the normal oral epithelium, including functionally different cell sub-populations: stem cells, transient amplifying cells and terminally differentiated cells [10]. Based on the ability of normal epidermal stem cells to rapidly adhere to basement membrane molecules such as collagen IV due to their differential expression of specific integrins [11], an adhesion assay for their isolation and enrichment was successfully developed in both human [11], murine [12], and rabbit normal epithelium [9]. We have adopted this method of isolating stem cells in normal epithelia for enrichment of TICs in OSCC, and determined whether a sub-population of cancer cells containing putative TICs could be separated over time according to adhesiveness to collagen IV. We report here, for the first time to our knowledge, that sub-populations of cells enriched for TICs, with increased self-renewal and higher tumourigenic potential could be isolated within 10 min using the method of rapid adhesiveness to collagen IV in OSCC.

2. Materials and methods

2.1. Cell culture and generation of primary cells from human samples

OSCC cell lines (Cal, H357 and CaLH3), human dysplastic oral keratinocyte (DOK) cell line and primary oral cancer keratinocytes isolated from patients with OSCC (P1 and P2) were used in this study. Written consent was obtained from all patients, and the project was approved by the Regional Committee for Ethics in Research in West Norway.

2.2. Adhesion to collagen IV

Tissue culture dishes (100 mm, Nunc, Denmark) were coated evenly with 10 μg/mL human collagen IV (Sigma, St. Louis, MO, United States of America (USA)) diluted in 10 mM acetic acid. Single cell suspensions in routine culture medium were allowed to attach to the collagen IV coated dishes in the incubator. Cells that attached within 10 min were collected after trypsinisation and referred to as rapid adherent cells (RAC). Cells that remained unattached within first 10 min were transferred to a new collagen IV-coated dish for additional 30 min in the incubator. Cells that adhered within this period were collected as middle adherent cells (MAC). Remaining unattached cells were then again transferred into a new collagen IV-coated dish for next 4 h. Adherent cells in this time period were collected and referred to as late adherent cells (LAC).
Un-fractionated cells that were not separated according to their adhesive properties served as controls.

2.3. Colony and sphere formation assays

Five hundred cells per well were seeded in 6-well plates (Nunc) in 3 mL complete culture medium. After 7–10 days, the wells were examined microscopically and stained with 0.5% crystal violet (Sigma). Colonies were manually counted. For sphere formation assay, 500 cells per well were seeded in 24-well plates (Nunc) with 500 μL routine culture medium containing 1% methylcellulose (Sigma). After 7–14 days, wells were examined microscopically and viable spheres were manually counted. Colony forming efficiency (CFE, %) and sphere forming efficiency (SFE, %) were calculated using the following formula: (average of colonies or spheres per well/500) × 100.

2.4. Tongue xenotransplantation in NOD-SCID mice

NOD-SCID 6–8 week old mice were used and maintained in an isolation facility under a pathogen free environment with standard 12/12 h day and night cycle. RAC, MAC and LAC cells isolated from P1 and P2 were suspended in 50 μL Matrigel (BD Biosciences) and transplanted into the tongue of mice (n = 91). All mice in each group were sacrificed at the same time. Tumour formation and loco-regional lymph node metastasis were assessed. All animal procedures were approved by the Norwegian Animal Research Authority.

Cell diameter measurement, cell migration assay, immunohistochemical staining, RNA extraction, polymerase chain reaction (PCR) array, quantitative PCR (qPCR), western blot, flow cytometry and cell cycle analysis are described in Supplementary information.

3. Results

3.1. RAC cells were smaller, more homogenous and had increased self-renewal ability

Quantification of the cell diameter showed that RAC cells were significantly smaller than MAC and LAC (Fig. 1A), both in oral dysplastic and OSCC cell lines, as well as in primary OSCC-derived cells. RAC cells displayed a regular round shape and were more homogenous when compared with MAC and LAC that were bigger, heterogeneous and brighter, as seen under bright field microscopy (Fig. 1B). Assessment of the in vitro self-renewal ability showed that RAC had significantly higher clonogenicity when compared with MAC, LAC.
and control cells for oral dysplastic cells, most of the OSCC cell lines, and for primary OSCC-derived cells (Fig. 1C and S). Sphere formation assay showed that RAC formed higher number of oro-spheres than MAC, LAC and control cells in OSCC cell lines (Fig. 1D).

3.2. RAC had increased in vivo tumourigenic and metastatic ability in primary OSCC-derived cells

A total of 91 mice were injected with different cell subpopulations isolated from two patients with OSCC, at serial dilutions (100, 1000 and 10,000 cells). RAC showed increased tumour initiating ability when compared to MAC and LAC (Fig. 2A and Table S1), while MAC and LAC had lower tumour initiation ability, and this was more obvious at higher dilutions. Tumour growth curves showed that RAC induced the biggest tongue tumours with the fastest tumour growth (Fig. 3). Of note, only tumours developed by RAC exhibited small islands of invading cells at the tumour front (Fig. 2B – a, d, g and j), while MAC (Fig. 2B – b, e, h and k) and LAC (Fig. 2B – c, f, i, and l), when they developed tumours, had a less aggressive pattern of invasion, more of the ‘pushing’ type tumour front, with no single cells or small islands invading the surrounding musculature. Examination of the loco-regional lymph nodes (Fig. 2D) demonstrated the presence of p53-positive human cells at a higher rate in the lymph nodes of mice injected with RAC (Fig. 2D – a and d), than in the mice injected with MAC (Fig. 2D – b and e) or LAC (Fig. 2D – c and f).

3.3. RAC showed higher expression of stem cell-related markers

Flow cytometry analysis of integrin β1 expression, a known stem cell marker in normal epidermis and a collagen IV receptor when heterodimerised with integrin α2
[11], revealed a significantly higher expression in RAC than MAC and LAC in all OSCC cell lines and primary cells (Fig. 4A and B). Immunohistochemical staining for P75NTR, a surface marker more recently related with stemness in normal oral epithelium showed higher expression in RAC than MAC and LAC for all cell lines (Fig. 4C). Western blot for a self-renewal related molecule, BMI1, revealed a higher level in RAC compared to MAC or LAC (Fig. 4D). To characterise more precisely the expression of stem cell-related molecules in the three cell sub-populations, 84 genes related to stem cell properties were analysed using a PCR array in primary OSCC-derived cells (P1). Unsupervised hierarchical clustering revealed distinct separation of RAC from LAC (Fig. 5A). In addition, MAC also clustered separately from LAC (Fig. 5B), but not from RAC (Fig. 5C). Pair-wise significance analysis of microarrays (SAM) analysis showed that genes related to the stem cell properties were differently expressed in RAC and MAC compared to LAC (Table S2). Compared to LAC, RAC were found to have significantly (FDR = 0) up-regulated expression of ABCG2, CCND2, PPARG, FGF2, DVL, RPL13A, GJA1 and GDF (Table S2). Differential gene expression pattern between RAC, MAC and LAC was further independently verified by quantitative real-time PCR (qRT-PCR) using TaqMan assays. In line with the PCR array results, RAC expressed significantly higher mRNA level of FGF2, PPARG, CCND2, TERT and RPL13A (Fig. 5D) than LAC. The expression of BMP1 was found to be significantly lower in RAC than LAC (Fig. 5D). Furthermore, RAC expressed significantly higher levels of NGFR and POU5F1 than LAC (Fig. 5E), however, no significant differences of CD44, BMI-1, and PDPN mRNA expression were found in RAC, MAC and LAC (Fig. 5E).

3.4. RAC displayed migratory behaviour and higher viability than FACS sorted CD44 high cells

Flow cytometry analysis after staining the three cell sub-populations for DNA content showed an increase in the proportion of cells in G2-phase of the cell cycle in RAC compared to MAC and LAC for all cell lines (Fig. 6A). Transwell migration assay showed that RAC had significantly higher rate of migration than MAC and LAC, consistent for all cells examined in the study (Fig. 6B and C).

When compared with TICs isolated based on the established method of FACS sorting for CD44 [13], the viability of RAC was significantly higher than that of CD44 high cells (Table 1).
4. Discussion

Many studies have showed that extracellular matrix (ECM), as an essential component of both normal and cancer stem cell niches, plays a crucial role in maintaining stem cell properties [14]. Collagen IV is an important ECM component and its expression pattern in the basement membrane of OSCC was associated with cervical lymph node metastasis as an important predictor of the metastatic aggressiveness of OSCC [15]. The present study describes, for the first time to our knowledge, the use of a simple, non-invasive and functional method to yield viable TICs in OSCC, based on rapid adherence to collagen IV. We show here that OSCC cells that adhered to collagen IV coated plates within 10 min displayed a number of stem cell properties, including small cell size, high in vitro self-renewal ability, high in vivo tumour initiating ability, high expression of stem cell markers as well as a high proportion of cells in the G2 cell cycle phase and high migratory capability. Previous studies suggested that cell size was related to cell phenotype, cell differentiation and proliferative potential in human keratinocytes [16]. The finding that RAC were smaller in size and showed higher expression of stem cell-related markers is in line with the previous study on human corneal epithelium which showed that small cells were expressing stem cell-associated markers and the small cell size represented one of the important stem cell properties while larger cells were more differentiated cells [17]. From the stem-cell markers assessed in this study, integrin \( \beta_1 \) is of particular importance. It is well known that integrin \( \beta_1 \) functions as the major receptor for basement membrane components such as collagen IV when heterodimerised with integrin \( \alpha_2 \), and laminin 5 when heterodimerised with integrin \( \alpha_3 \) [18]. Early studies showed that FACS for cells highly expressing integrin \( \beta_1 \) can be used to isolate normal human epidermal stem cells from transit amplifying cells, since the cells with the highest level of integrin \( \beta_1 \) expression showed the greatest colony formation ability [11]. In corroboration with these studies performed on normal epithelium, the present study shows that in a malignant epithelium the cells displaying the highest level of integrin \( \beta_1 \) (RAC) also had the highest colony formation.
In addition to increased clonogenicity and sphere formation ability, we show that the brightest integrin β1 cells (RAC) have also the highest tumour initiating abilities. In the present study we used orthotopic tongue xenotransplantation in NOD-SCID mice. This model was chosen because the human xenotransplantation cells are allowed, in the immunocompromised animal models, to develop and form tumours that mirror the phenotypic heterogeneity of the original human tumour, thus being still considered the ‘gold standard model’ for testing tumour initiating properties [19]. Despite the fact that the most common model for tumourigenesis testing is the subcutaneous xenotransplantation model, we have chosen the orthotopic tongue model, since in this way we could evaluate the loco-regional lymph node metastatic ability, this type of metastasis being the most common in OSCC.

Additional stem cell traits were also shown in this study for the RAC cells, including the higher proportion of cells in G2 phase of the cell cycle, consistent for all cell lines tested. Human malignant epithelial cells with stem-like properties were shown previously to spend a longer time in G2 phase in order to escape apoptosis by activating G2/M checkpoint proteins and thus provide more time for repair of DNA damage [13]. We did not investigate the ability of RAC cells to resist apoptosis compared to MAC and LAC, but based on the cell cycle analysis it is expected that they will show increased resistance. However, this needs to be further tested.

More recently, EMT features were attributed to stem-like cells in carcinomas, including OSCC [20]. We have assessed here the in vivo migratory/metastasising potential of the three subpopulations of RAC, MAC and
LAC by quantifying their presence in the cervical lymph nodes 74 days after tongue xenotransplantation. To visualise the human malignant cells present in the lymph nodes we have used IHC staining with a human specific antibody for mutated p53 (the primary cells injected in mice were chosen after sequencing of \textit{TP53} and only cells derived from \textit{TP53} mutated tumours were used for this purpose), that specifically identifies these cells and not mouse cells. Our results showed that RAC cells have the highest \textit{in vivo} migratory/metastasising potential since the rate of cervical lymph nodes with scattered p53-positive human cells was higher in mice injected with RAC than in mice injected with MAC or LAC, fulfilling thus one more criteria recently linked to stemness in carcinomas.

Although isolated from tumours of the same histological type and clinical staging, \textit{in vivo} tumour formation and especially the metastasising ability varied between the two primary cell lines used in the study. For example, no tumours were formed when 100 cells from MAC P2 cells were injected in six mice (Fig. 2A, Table S1). Of interest, we have detected p53-positive human migratory cells in the lymph nodes of five of these mice (Fig. 2C and D, Table S1). This could be a methodological bias, since for comparison reasons we have sacrificed all mice at the same time point, when the RAC tumours were too large and impaired the well-being of the mice. It might be that at this time point the primary tumours formed by the 100 MAC cells injected were too small to be detected, although migratory p53-positive human cells could be histologically detected in the lymph nodes. This might be taken as an indication that migration of malignant cells to the lymph nodes happens quiet early in the process of tumourigenesis, or at least in our experimental model of tumourigenesis.

Taken together, the findings presented here show that the cells rapidly adherent to collagen IV are enriched for cells with stem cell-like properties, and suggest that the method based on the adhesiveness of collagen IV provides a low cost, non-invasive, functional alternative to FACS for isolation and enrichment of TICs in OSCCs. The sub-population being rapidly adherent to collagen IV could be further used to explore stem cell and tumour initiating properties, improving our knowledge of OSCC biology.

\textbf{Conflict of interest statement}

None declared.
Acknowledgements

We thank Gunnvor Øijordsbakken and Edith Fick for excellent technical assistance. We also thank Molecular Imaging Centre (MIC) for outstanding facilities. This study was supported by The Norwegian State Educational Load Fund (Quota Programme), Meltzer’s fund (project no. 804513, DS), Bergen Medical Research Foundation (DEC, 2010/11 and JW, 2010) and The Norwegian Cancer Association (2245585, 3294890).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2014.09.010.

References