KGF suppresses $\alpha_2\beta_1$ integrin function and promotes differentiation of the transient amplifying population in human prostatic epithelium

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Summary

Prostate epithelial stem cells are self-renewing cells capable of differentiation into prostate epithelium, and are thought to contribute towards both benign and malignant conditions in the human prostate. We have previously demonstrated that prostate epithelial basal cells express high levels of integrin $\alpha_2\beta_1$ and this population can be subdivided into stem ($\alpha_2\beta_1^{hi}$ CD133$^+$) and transient-amplifying population (TAP) cells ($\alpha_2\beta_1^{hi}$ CD133$^-$). However, the molecular mechanism(s) controlling the commitment and regulation of these cells towards differentiated epithelium remains unclear. Here, we demonstrate that β_1 integrin function is required for the maintenance of basal prostatic epithelial cells and suppression of its function by either methylcellulose or, more specifically, β_1-blocking antibody (80 μg/ml) induces differentiation, with associated expression of the differentiation-specific markers prostate acid phosphatase (PAP) and cytokeratin 18 (CK18). Keratinocyte growth factor (KGF), a stromal-derived growth factor, has previously been implicated in prostate organogenesis using in vitro tissue recombination experiments. We show that treatment with KGF (10 ng/ml) potently induces epithelial differentiation with concomitant suppression of $\alpha_2\beta_1$ integrin expression as well as the induction of androgen receptor expression. Specifically, p38-MAPK appears to be involved and the presence of SB202190, a p38 inhibitor, significantly blocks KGF-induced differentiation. Furthermore, the expression of the high-affinity receptor tyrosine kinase to KGF (FGFR2) is predominantly detectable in $\alpha_2\beta_1^{hi}$ CD133$^-$ TAP cells when compared with stem cells ($\alpha_2\beta_1^{hi}$ CD133$^+$), which would therefore be relatively unresponsive to the differentiating effect of KGF. Taken together, using a human primary culture model, we have demonstrated key roles for interactions between KGF and integrin-mediated function in the regulation of prostate epithelial differentiation.

Key words: Stem cells, Transient amplifying cells, Prostate, Epithelia, β_1 integrin, p38, KGF

Introduction

Prostate cancer and benign prostatic hyperplasia (BPH) are major clinical issues, with aberrant stem cell growth considered to be a key mechanism behind the formation of both diseases (Collins and Maitland, 2003; Feldman and Feldman, 2001; Reya et al., 2001). Our group has previously demonstrated that the prostate epithelial basal cell population expresses high levels of integrin $\alpha_2\beta_1$ (Collins et al., 2001). We have subsequently demonstrated that this basal cell population can be further defined into stem (CD133$^+$ $\alpha_2\beta_1^{hi}$) and transient-amplifying population (TAP) cells (CD133$^-$ $\alpha_2\beta_1^{hi}$). This has provided the opportunity to characterise the molecular mechanisms involved in the regulation of adult prostate stem cells through various degrees of differentiation and to examine how these processes may contribute to these diseases of the prostate. This stratification of the basal epithelial population is a landmark finding in the field of prostate developmental biology as the majority of work has focused on the stem cell with very little data on the role of the TAP. Indeed the TAP is thought to play an important role in the development of androgen-independent prostate cancer (Feldman and Feldman, 2001) and underlines the importance for its investigation. This study aims to characterise some of the mechanisms involved in regulating the prostate TAP through differentiation.

Three histologically distinct cell types have been identified in prostate epithelium: basal, secretory luminal and neuroendocrine. The luminal cells express markers of differentiation: prostate-specific antigen (PSA), androgen receptor (AR), cytokeratin 18 (CK18) and prostate acid phosphatase (PAP) (Bonkhoff et al., 1994a; Prins et al., 1991; Wernert et al., 1987). During castration, the adult prostate gland undergoes involution as the luminal epithelial cells are dependent upon androgen for their survival (Bonkhoff and Remberger, 1996; Isaacs, 1984; Kyprianou and Isaacs, 1988). Furthermore, the basal layer, which contains the stem cell population, is multipotent giving rise to all the epithelial derivatives (Bonkhoff et al., 1994a; Collins et al., 2001; Robinson et al., 1998). Consequently, a stem-cell model for the prostate epithelium was proposed: the basal compartment contains a small population of slowly proliferating stem cells, which give rise to an intermediate and more rapidly cycling TAP cells. The TAP cells, which are androgen responsive but

not dependent upon androgen for survival, have limited proliferative potential and subsequently undergo terminal differentiation into androgen-dependent luminal secretory cells (Hudson et al., 2000; Isaacs and Coffey, 1989). In the cancer stem-cell model, abnormal regulation of proliferation and differentiation of the stem cell/TAP cell are thought to be key mechanisms of pathogenesis (Reya et al., 2001).

Prostatic development is dependent on the interplay between the epithelium, mesenchyme and extracellular matrix (ECM) (Cunha et al., 1991). A number of candidate regulators of prostate epithelial differentiation have been identified including Notch1, TGF, IGF, HGF/cMET, p63 and ERβ (Byrne et al., 1996; Culig et al., 1996; Imamov et al., 2004; Nylander et al., 2002; van Leenders et al., 2002; Wang et al., 2004). Members of the fibroblast growth factor (FGF) family have also been implicated in the control of prostate epithelial development. This study explores the particular role of keratinocyte growth factor (KGF/FGF7) which has been implicated in the androgen regulation of murine prostate organogenesis (Alarid et al., 1994; Cunha, 1996; McKeehan, 1991; Sugimura et al., 1996). Tissue recombination experiments with normal (wild-type) urogenital sinus mesenchyme and testicular feminization epithelium (containing no functional AR) show that androgens induce AR-positive mesenchymal cells to secrete paracrine agents, such as KGF, which in turn regulate the growth and differentiation of prostate epithelium (Cunha, 1996; Cunha and Young, 1991; Donjacour and Cunha, 1995; Sugimura et al., 1996). The exact mechanisms by which AR, KGF and the ECM interact in deciding cell fate in the prostate remains ill defined and this is examined in human prostate epithelium by this study.

The stem cell niche is considered to be a microenvironment consisting of a subset of cells and extracellular substrates that continuously maintain the stem cell population and controls their self-renewal and differentiation in vivo (Schofield, 1978). Integrins are important cell surface adhesion molecules that bind cells to the ECM and are involved in maintaining stem cells in their niches in a number of tissues (Jones et al., 1995; Li et al., 1998). In keratinocytes, binding of β_1 integrins to the extracellular matrix inhibits terminal differentiation (Adams and Watt, 1989; Levy et al., 2000) and maintains the epidermal stem cell compartment in vitro (Zhu et al., 1999). Using a monoclonal antibody to the β_1 integrin, the proportion of keratinocyte β_1 integrins occupied by ligand can regulate the initiation of terminal differentiation (Watt et al., 1993). This finding is of particular relevance to the prostate, as epithelial stem cells and TAP cells are rich in $\alpha_2\beta_1$ integrin expression (Collins et al., 2001) – suggesting that integrin β_1 may be a key regulator of prostate epithelial cell fate. Furthermore, mitogen-activated protein kinases (MAPK) pathways have also been implicated in the regulation of integrin expression and function (Tanaka et al., 2002; Zhu et al., 1999) identifying a possible mechanism of crosstalk between KGF pathways and integrin expression on prostatic epithelial cells.

In this study, we demonstrate that blocking the β_1 integrin in vitro induces differentiation of the $\alpha_2\beta_1^{hi}$ integrin prostate epithelial cell population. Furthermore, we show that KGF induces differentiation by negatively regulating the $\alpha_2\beta_1$ integrin expression on these cells. Within this basal cell population, it is the TAP cells ($CD133^-$ $\alpha_2\beta_1^{hi}$) that show significant KGF receptor expression when compared with stem cells ($CD133^+$ $\alpha_2\beta_1^{hi}$), in keeping with a model for the preservation of the stem cell population throughout life.

Results

Methylcellulose suspension of prostatic epithelial cells induces differentiation

The effect of abolishing all ECM interaction, using MC cell suspension, on the $\alpha_2\beta_1^{hi}$ cell population was examined by monitoring expression of PAP. Incubating these cells in an MC suspension for 24 hours resulted in a potent induction of PAP expression (Fig. 1A,B). Cytoplasmic expression of PAP was confirmed by immunofluorescence and, upon higher magnification, PAP expression localised in a distinct vesicular pattern in keeping with the secreted nature of this protein (see inset Fig. 1A). Counting only the DAPI-stained cells after 24 hours, the number of cells showing PAP expression increased from a mean of 8.8% (control group) to 89% in the MC-treated group of $\alpha_2\beta_1^{hi}$ cells (P=0.0015, two-sided paired t-test).

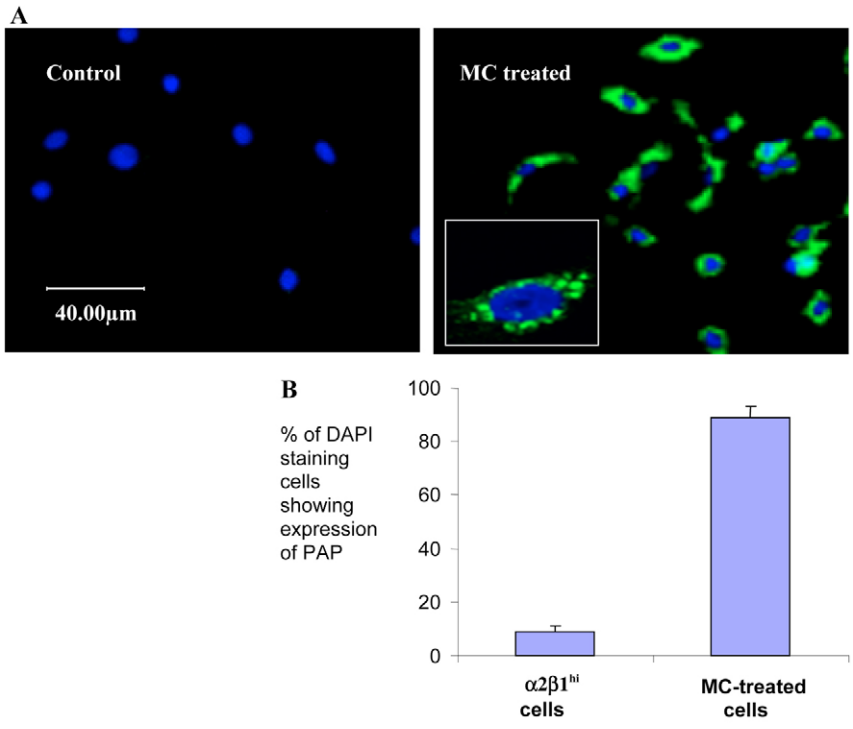

Fig. 1. Methylcellulose (MC) suspension induces differentiation. (A) Control $\alpha_2\beta_1^{hi}$ and MC-treated $\alpha_2\beta_1^{hi}$ prostatic epithelial cells stained for PAP (FITC/green) and the nuclear stain DAPI (blue). Inset is at increased magnification (×800). (B) Bar chart summarising PAP expression in $\alpha_2\beta_1^{hi}$ cells. Data are mean ± s.e.m. of three experiments.

Specific inhibition of the β_1 integrin function induces basal prostatic epithelial cell differentiation

Using a mouse monoclonal IgG1-blocking antibody, against the integrin β_1 subunit, expression of prostate differentiation markers was assessed by FACS analysis. The blocking antibody and the fluorochrome conjugates used to measure the markers of differentiation were controlled for by including isotype controls (Fig. 2A). The results for the effect on the expression of the differentiation markers PAP, CK18 and AR after blocking of β_1 integrin on the $\alpha_2\beta_1^{hi}$ epithelial cells for 1 hour are summarised in Fig. 2B. Blocking β_1 integrin on the basal prostatic epithelial cells resulted in an increase in the mean number of cells expressing PAP from 4% to 62.5% (P=0.001, two-sided paired t-test) and CK18 from 3% to 35% (P=0.0014, two-sided paired t-test). However, despite the increased PAP and CK18 expression, there was no increase in the mean expression of AR (3.5% in the $\alpha_2\beta_1^{hi}$ group and 3% in the β_1-integrin-blocked group). The effect of blocking β_1 integrin on the $\alpha_2\beta_1^{hi}$ cells was also assayed by immunofluorescence (Fig. 2C), which confirmed the induction of PAP and CK18 expression. Consistent with the results from the FACS analysis, AR induction was not detected. The PAP expression was observed in a punctate pattern of expression within the cytoplasm (consistent with the expected vesicular distribution) and CK18 was seen to stain diffusely throughout the cytoplasm.

Blocking β_1 integrin leads to a differentiated cellular morphology and increases the number of S-phase cells

Distinct morphological differences are seen in the prostate epithelium, when comparing the basal layer with the differentiated luminal cells (Robinson et al., 1998). We used scanning electron microscopy (SEM) to study morphological changes associated with the blockade of β_1 integrin function on the $\alpha_2\beta_1^{hi}$ epithelial cells (Fig. 3A). The $\alpha_2\beta_1^{hi}$ prostatic epithelial cells were seen as flat sheet-like cells devoid of cytoplasmic processes; however, following treatment with the blocking antibody, a distinctly spherical cellular phenotype was observed, which demonstrated a higher degree of protocytoplasmic extensions at the cell-substratum junction.

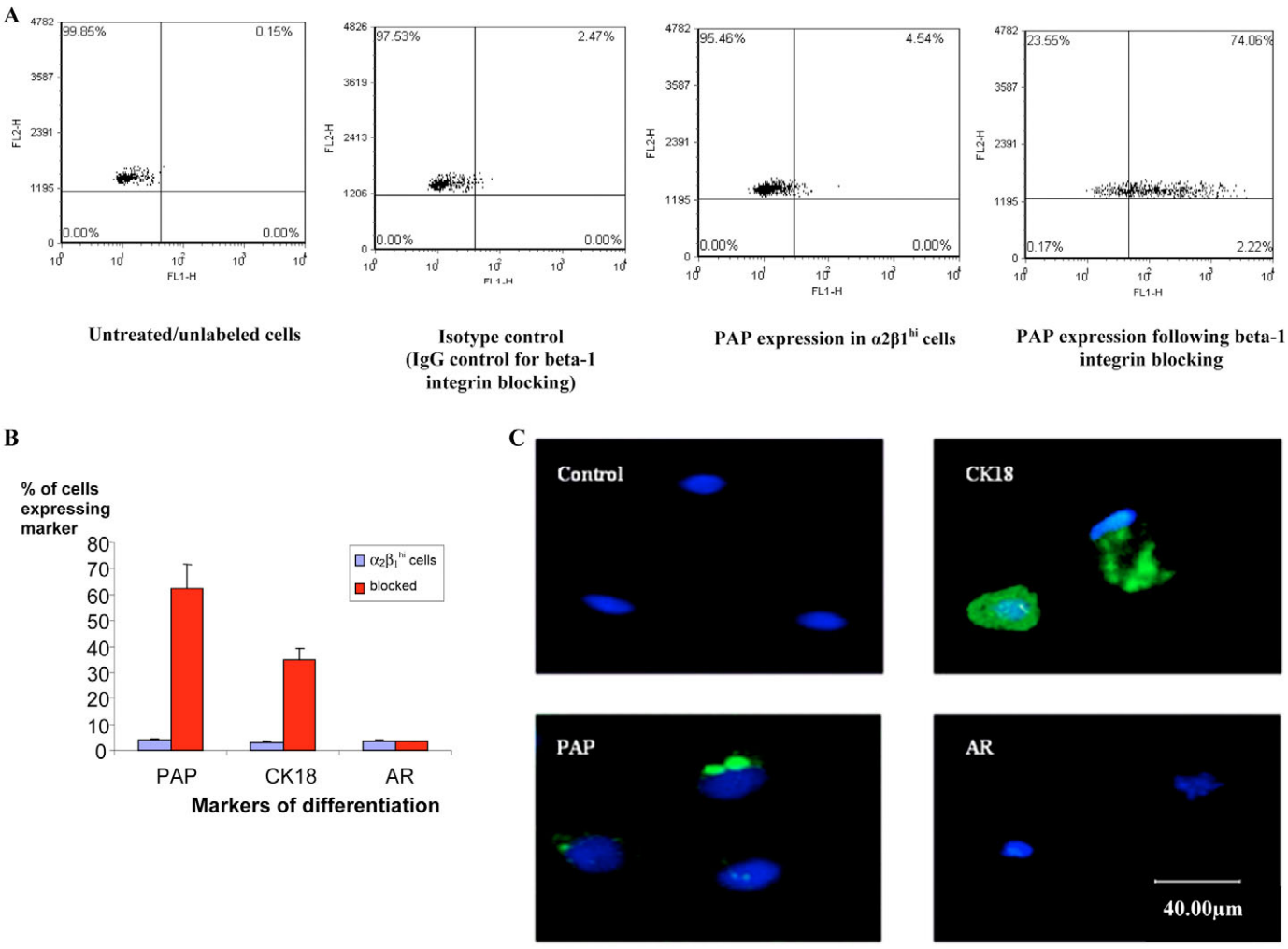

Fig. 2. Blocking β1 integrin function induces differentiation. (A) Dot plots of PAP expression following blocking with anti-β1-integrin antibody. Controls were set at 3%. (B) Bar chart summarising the effect of blocking β_1 integrin on the expression of differentiation markers. Data are mean ± s.e.m. of three experiments. (C) Expression of differentiation markers CK18, PAP and AR (FITC/green) in $\alpha 2\beta 1^{hi}$ cells treated with β_1-blocking antibody. Cells were counterstained with the nuclear stain DAPI (blue).

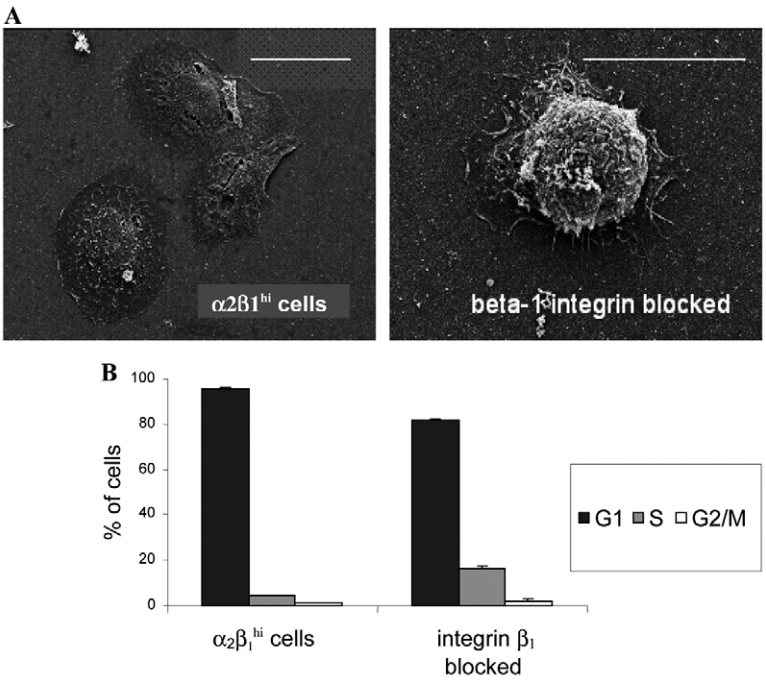

Fig. 3. Changes in cell morphology and cell cycle with blockade of the integrin β1. (A) Scanning electron microscopy of $\alpha2\beta1^{hi}$ cells treated with the β_1-blocking antibody. (B) Effect on the cell cycle following $\alpha2\beta1^{hi}$ cell treatment with β_1-blocking antibody. Data are mean ± s.e.m. of three experiments. Bars, 20 μm.

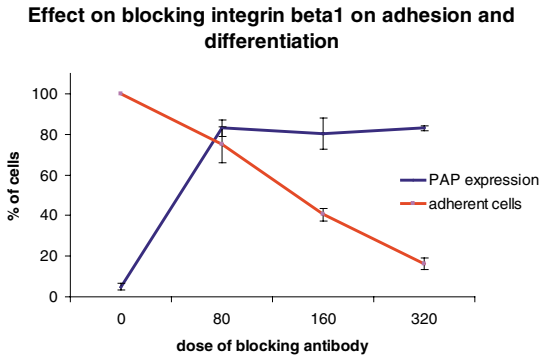

Fig. 4. Relationship between the dose of β1-blocking antibody and $\alpha2\beta1^{hi}$ cell differentiation and adhesion. Effect of the β1-blocking IgG on the number of cells adhering to collagen type-1 and expression of the differentiation marker PAP. Data are mean ± s.e.m. of three experiments.

The stem cell model of tissue homeostasis describes stem cells initially developing into an intermediate amplifying population (TAP) before undergoing terminal differentiation. There is considerable uncertainty regarding the state of these intermediate cells; is differentiation associated with proliferation – as would be expected in the TAP? To determine the effect of β_1 integrin blockade, cell cycle analysis using flow cytometry was used, demonstrating that a mean of 96% of $\alpha_2\beta_1^{hi}$ epithelial cells are in the G1 phase and 4% are in S-phase (Fig. 3B). Basal epithelial cells treated with blocking β_1 integrin antibody resulted in an increase in proliferation: 82% of cells were seen in G1 phase with a concurrent increase in S-phase to 16% (P=0.016, two-sided paired t-test).

Effect of β_1 integrin blockade on prostatic epithelial $\alpha_2\beta_1^{hi}$ cell adhesion and differentiation

Our hypothesis supports the idea that blocking the β_1 integrin results in loss of adhesion from the basal membrane and subsequent migration towards the lumen while undergoing differentiation. To test this hypothesis we examined whether the effects of blocking β_1 integrin on adhesion to ECM and differentiation are inter-related. When examining the effect on $\alpha_2\beta_1^{hi}$ cell adhesion to collagen type-1 using an increasing concentration of β_1-integrin-blocking antibody we observed an increased effect on impairment of cell adherence to ECM (Fig. 4). There was an inverse, dose-dependent, linear relationship on the number of cells adherent to collagen type-1 following incubation with β_1-blocking antibody for 1 hour (r=0.98). Interestingly, this was mirrored with a dose-dependent decrease in α_2 integrin (specific subunit ligand for collagen type-1) expression measured by FACS (data not shown). The effect of β_1-blocking antibody on differentiation was examined using immunofluorescence (Fig. 4). In contrast to the dose-dependent relationship between α_2 integrin expression and adherence function to collagen type-1, a drastic induction of PAP was observed without any further increase in the number of cells showing PAP expression despite increasing concentrations of β_1-blocking antibody (the slightly higher portion of cells expressing PAP, compared with the FACS-based assay in Fig. 2B, probably represents differences in detection by immunofluorescence and the staining protocol).

KGF induces downregulation of the $\alpha_2\beta_1$ integrin on basal epithelial cells and stimulates their differentiation

KGF has been implicated to be important during organogenesis of the murine prostate (Cuhna et al., 1991) and we aimed to test its role in human prostate homeostasis. Using flow cytometry, isolated $\alpha_2\beta_1^{hi}$ epithelial cells treated with KGF (10 ng/ml), were examined for the expression of differentiation markers. Increases in the number of cells showing expression of PAP, CK18 and AR were observed: 34% (P=0.015), 35% (P=0.015) and 39% (P=0.009) respectively (Fig. 5A). In addition, a concurrent downregulation of $\alpha_2\beta_1$ integrin expression was seen with KGF treatment (P=0.017). In human prostate cells, KGF signals predominantly via p38-MAPK (Mehta et al., 2001). Hence, the specific p38 inhibitor (SB202190; 5 μM) was applied to test whether it would influence KGF-induced differentiation. KGF-induced expression of multiple differentiation markers was potently abolished by the presence of SB202190 (P<0.05; Fig. 5A). To test for the presence of a functional AR, triple-label immunofluorescence studies confirmed PSA expression in addition to AR induction by KGF (Fig. 5B). KGF-treated cells demonstrated a perinuclear pattern of AR expression, and the detection of PSA colocalisation supported the presence of a functional AR. To determine which subpopulation of the $\alpha_2\beta_1^{hi}$ epithelial population was responsible for differentiation, KGF receptor (KGFR) mRNA was studied in the $\alpha_2\beta_1^{hi}$ $CD133^+$ (stem) and $\alpha_2\beta_1^{hi}$ $CD133^-$ (TAP) cells. RT-PCR confirmed

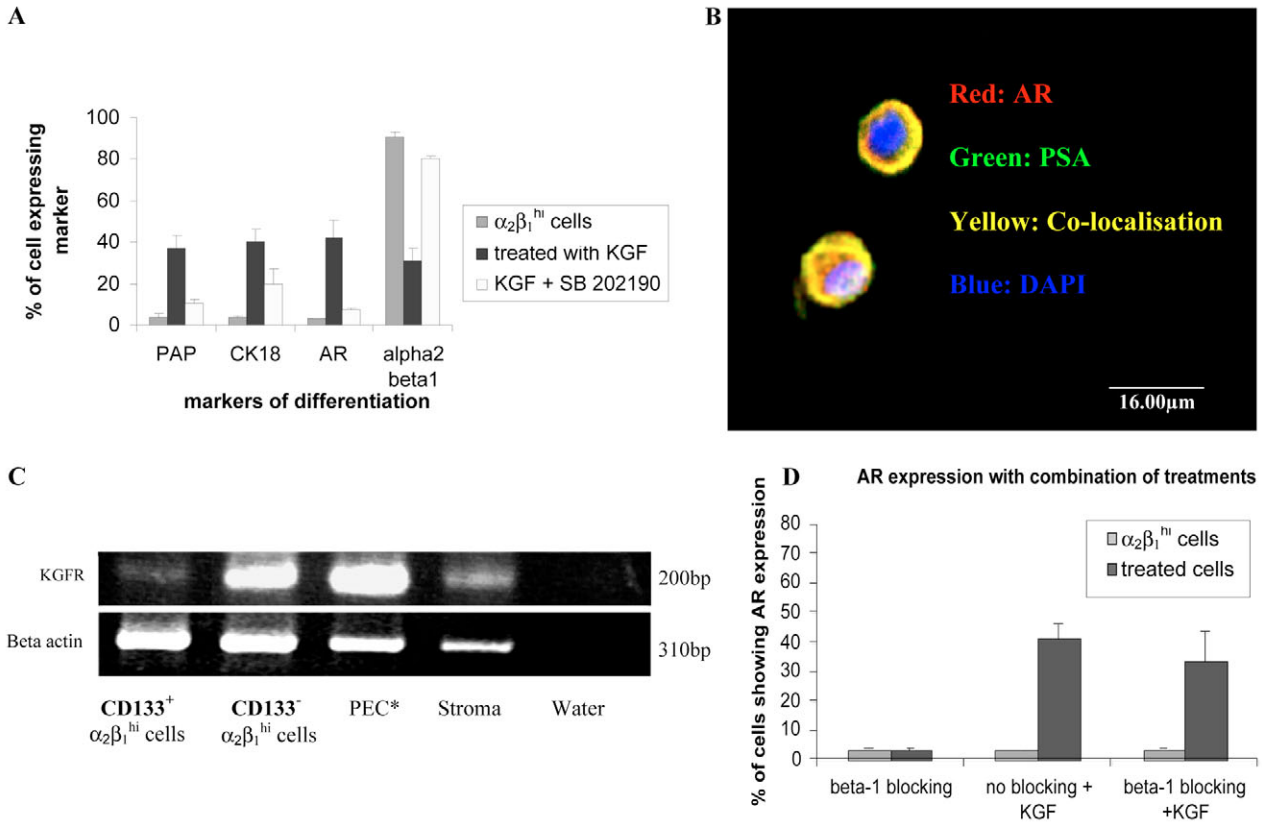

Fig. 5. Effect of KGF treatment on α2β1^{hi} cells. (A) Bar chart summarising the effect of KGF on expression of differentiation markers PAP, CK18 and AR (measured by FACS) in α2β1^{hi} cells. (B) Triple immunofluorescence study of α2β1^{hi} cells treated with KGF. AR, TRITC/red; PSA, FITC/green; DAPI, blue. Colocalisation is indicated in yellow. (C) RT-PCR for KGFR mRNA expression in primary prostate cell sub-populations. *PEC, primary culture of epithelial cells following subtraction of $\alpha_2\beta_1^{hi}$ basal cells. β-actin levels were detected as a control (lower gel). (D) Summary of FACS analysis of AR expression in α2β1^{hi} cells untreated or treated with combinations of KGF and the β1-blocking antibody. Data are mean ± s.e.m. of three experiments.

expression of KGFR in $\alpha_2\beta_1^{hi}$ cells predominantly expressed within the α2β1^{hi} CD133^{-} transient amplifying population. KGFR expression was also detected in stroma and primary culture of the differentiated epithelium (PEC*, epithelial cells following subtraction of $\alpha_2\beta_1^{hi}$ basal cells) (Fig. 5C).

Our data demonstrated that KGF induces expression of PAP and CK18, with β_1 integrin closely involved. However, KGF stimulation, unlike the blockade of β_1 integrin function, has the additional effect of inducing the expression of AR. We tested the hypothesis that downregulation of the β_1 integrin has a permissive effect on KGF function with regard to its unique effect on AR expression. β_1 integrin blocking in combination with KGF (33.5±6.9%) however did not lead to an additive increase in AR expression, when compared with KGF treatment alone (41.7±4.7%) (Fig. 5D).

Discussion

Single-cell suspension of human keratinocytes in methylcellulose induces terminal differentiation (Green, 1977) as it is the cell adhesion to the ECM that maintains the stem cell population (Adams and Watt, 1989; Levy et al., 2000; Watt et al., 1993). In parallel to this finding in keratinocytes, we have now demonstrated that methylcellulose cell suspension can induce prostatic basal epithelial cell differentiation. More specifically, using a blocking antibody, we demonstrate that β_1 integrin expression negatively regulates epithelial cell differentiation. Mouse prostate organogenesis is, in part, under the hormonal control of KGF (Sugimura et al., 1996). We investigated the role of KGF in regulating human prostatic epithelium and demonstrated it to be a key mechanism in differentiation, acting, in part, through the p38-MAPK pathway. In our model, we demonstrate that KGF acts on the TAP cells of the basal layer to induce downregulation of $\alpha_2\beta_1$ integrin expression and causes subsequent differentiation.

Different integrins show an ability to interact with a number of ECM substrates and the $\alpha_2\beta_1$ integrin shows the greatest specificity for collagen type-1. The β_1-blocking antibody has a dose-dependent inhibition on adhesion of prostatic epithelial basal cells onto collagen type-1. Interestingly, when looking at its effect on differentiation we observed a switch-like induction of differentiation, with no further inducible expression of differentiation markers. It appears that the relationship between the degree of cellular adhesion to the ECM and the degree of differentiation are not mutually exclusive. This would allow adherent cells to undergo differentiation. This observation is in keeping with studies looking at β_1 integrin expression and cell adherence: in keratinocytes, it is the absolute number of vacant β_1 integrin

receptors rather than their relative proportion that determines commitment differentiation (Levy et al., 2000).

In our model, the basal cells were treated with the β_1-blocking antibody for 1 hour and then re-plated onto collagen type-1. This suggests that commitment to differentiation happens rapidly and re-attachment to collagen type-1 does not appear to significantly reverse this cell fate. This is consistent with observations in vivo where the committed stem cell remains associated with the collagen-rich ECM environment whilst exiting the stem cell compartment (Spradling et al., 2001).

Blocking the β_1 integrin resulted in distinct morphological changes that are consistent with prostatic epithelial differentiation and organisation in vitro (Robinson et al., 1998). Multilayered primary prostate epithelial culture examined by SEM demonstrated underlying monolayers of flattened epithelial sheets with distinct spherical cells superimposed in the upper layer. Transmission EM showed the underlying cells to have sparse cytoplasm with prominent elongated nuclei whereas the upper layer had numerous secretory vacuoles, some containing granules, with less-prominent nuclei. These spherical cells have been shown to correspond with the expression of CK18, AR and PSA (Robinson et al., 1998).

The β1 integrin has been shown to play a crucial role in the regulation of progenitor neural cell proliferation and modulates ECM interaction by growth factors (Leone et al., 2005). The role of the β_1 integrin in regulating the proliferating population (TAP cells) of the prostate epithelial was examined. Cell-cycle analysis of the prostate epithelial basal cells following blockade of β1 integrin demonstrated an increase in the number of cells in the S-phase, implying that these cells are induced into proliferation. Autoradiographic studies of [^{3}H]thymidine uptake in prostate epithelial explants demonstrate that only the basal compartment is active in DNA synthesis (Bonkhoff et al., 1994b; Dermer, 1978). These proliferating cells within the basal compartment probably represent the TAP. The classically described hierarchical stem cell model of differentiation has the stem cell differentiating into a proliferating transit amplifying cell, before exiting the cell-cycle and undergoing terminal differentiation. It is thought that the stem cell can undergo asymmetric division to give rise to a cell committed to differentiation and a daughter cell to maintain the stem-cell pool (Spradling et al., 2001). This asymmetric division of stem cells is believed to augment the TAP and protect against the depleting effect of TAP cells undergoing terminal differentiation. Interestingly, in our studies, the changes in the cell cycle seen upon provoking differentiation suggest that the TAP cell itself may divide asymmetrically. We observed increased cell cycling into the S-phase in keeping with proliferation and yet we were also able to demonstrate concurrent differentiation. Asymmetric division of the TAP would give rise to a daughter cell committed to differentiation, which could exit the cell cycle to become a luminal cell, and also give rise to a second daughter cell for self-renewal, to help the stem cells replenish the TAP.

The effect of methylcellulose cell suspension on the basal epithelial prostate cells drives the majority of the cells into differentiation (89% of cells expressing PAP). However, there appears to be a distinct population that does not develop expression of PAP. This may represent a subpopulation of stem cells or TAP cells that are refractory to the effects of suspension and possibly demonstrates that the role of ECM is only a component of the elements, such as growth factors, involved in determining the fate of these cells. Upon examining the specific effects of blocking the β_1 integrin there is an increase in the proportion cell refractory to differentiation, suggesting selectivity of action in a subgroup of basal epithelial.

During prostate embryogenesis, AR-mediated mesenchymal stimulation induces paracrine signalling, via KGF, to regulate development and differentiation of mouse epithelial cells (Cunha, 1996; Cunha and Young, 1991; Donjacour and Cunha, 1995; Sugimura et al., 1996). Similarly we show for the first time that KGF is a regulator of human prostate epithelial basal cell fate. KGF leads to the downregulation of the $\alpha_2\beta_1$ integrin and subsequent differentiation. KGFR (FGFR2IIIb) expression in prostatic epithelial basal cells was confirmed, consistent with previous observations in normal adult prostate and during embryogenesis (Giri et al., 1999; Thomson et al., 1997). Specifically, KGFR expression appeared to be localised predominantly in $\alpha_2\beta_1^{hi}$ CD133$^-$ TAP cells, with little expression in $\alpha_2\beta_1^{hi}$ CD133$^+$ stem cells. The specific investigation of differentiation in the $\alpha_2\beta_1^{hi}$ CD133$^+$ or CD133$^-$ cells with respect to blockade of the β_1 integrin was not addressed, however, the data presented demonstrate the mechanistic action of KGF-induced differentiation, which is by downregulation of the α2β1 integrin. (The role of the β_1 integrin with particular references to the CD133$^+$ and CD133$^-$ population will become a focus for further studies.) The pattern of KGFR expression would be in keeping with the in vivo expression of KGF showing selectivity for the TAP cells, and not serve to deplete the stem-cell population that would be required throughout life. The effect of KGF was significantly suppressed by a p38-MAPK inhibitor. Our data showed that the blocking β1 integrin IgG was effective at inducing basal cell differentiation as determined by the expression of PAP and CK18, whereas AR expression was not induced. By contrast, KGF-induced prostate epithelium also demonstrated upregulated AR expression. Perinuclear localisation of the AR was observed; however, in the absence of androgens in this model it is not surprising that more marked nuclear translocations are not seen. In our examination of the effects of KGF in combination with blocking the β_1 integrin, there was no evidence of synergistic/additive effects on the induction of AR expression. It would appear that KGF-induced differentiation works, in part, by downregulating β_1 integrin, which forms only a component of this process, perhaps contributing to the morphological and migratory effects during differentiation. Furthermore the downregulation of the $\alpha_2\beta_1$ integrin may allow, as discussed above, a degree of selectivity in a basal-cell subpopulation: limiting the differentiation effects to the TAP. However, the functionality of the differentiating TAP cells, i.e. AR and PSA expression, are attributes derived from the direct effects of KGF.

The effects of KGF appear not to be limited to inducing cellular differentiation. Our data show the expression of KGFR persisting in the differentiated epithelial cells from primary culture (PEC*, Fig. 5C). This may reflect a role of KGF in maintaining the terminally differentiated cell population. Interestingly, the effects of KGF during embryogenesis in in vivo rat models, can mimic the effects of androgen, which is known to maintain the luminal cell population (Thomson et al., 1997). It would be interesting to see if in vitro and in vivo

blockage of KGF function results in involution of the prostate gland with only the basal cells persisting.

The ability to understand the factors that control transfer between different compartments of the prostate epithelium allows novel insights into prostate differentiation. Very little is known about the mechanisms that allow epithelial TAP cells to undergo terminal differentiation. In keratinocytes, transition from proliferation to differentiation is associated with regulation of the p53/mdm2 pathway. This switch is delayed in carcinoma cells (Dazard et al., 2000), underlining the importance of aberrant differentiation mechanism in carcinogenesis. This finding is of particular relevance to prostate carcinogenesis as abnormal differentiation of the TAP could explain the emergence of androgen-independent disease. The TAP cells are not dependent on androgen for survival and this proliferating population would be selected by androgen ablation therapy (Feldman and Feldman, 2001). The role of the KGF/p38 pathway in inducing expression and stimulation of the AR during differentiation of the TAP may have considerable significance in androgen independent disease. It has previously been suggested that growth factors, including KGF, IGF and EGF, can activate the AR via MAPK pathways (Culig et al., 1996; Culig et al., 1994; Franco et al., 2003; Planz et al., 2001) and this pathway has previously implicated in hormone-resistant prostate cancer (Lin et al., 2001; Uzgare and Isaacs, 2004; Uzgare et al., 2003). Interestingly, we observed that KGF/p38 induced PSA production, consistent with the phenomenon of crosstalk between the KGF and AR pathways. Clearly this represents a potential pathway for the development of androgen-independent prostate cancer, although its clinical significance remains to be tested.

Debate remains about the source of neuroendocrine (NE) cells in the prostate epithelium and this too represents a field for additional work. Are the TAP, luminal and NE cells derived from the same stem-cell lineage or do they represent different differentiation pathways? Although not specifically addressed in this study, it would be interesting to examine if the TAP population is able to give rise to NE cells under the effects of KGF or if there is commitment to NE cell fate at the stem cell level. In keratinocytes, cMyc promotes stem cell differentiation into TAP cells that are committed to sebaceous lineages (Arnold and Watt, 2001). Similarly in the prostate, TAP cells specifically destined to a specific epithelial cell type (luminal or NE cells) may exist in the prostate, whose fates are decided upon exiting the stem cell compartment. Indeed, the regulators of stem-cell commitment in the prostate remains undefined and studies to examine putative regulators, such as cMyc, would be of interest.

In summary, our data support a model where the $\alpha_2\beta_1$ integrin maintains the prostatic epithelial basal cell compartment, with its expression negatively regulated by stroma-derived KGF (androgen regulated) acting through p38-MAPK. These findings support the importance of β_1 integrin in epithelial cell differentiation across a number of tissues.

Materials and Methods

Tissue collection

Human prostatic tissue was obtained from 65 patients (age range 56-88 years) undergoing either transurethral transaction of the prostate (TURP) for BPH or cystoprostatectomy for bladder cancer. All tissue obtained was with patient consent and ethical approval, in addition there was confirmation of benign histology by a clinical pathologist.

Isolation of the $\alpha_2\beta_1^{hi}$ integrin (high) and CD133 population of prostatic epithelial cells

The $\alpha_2\beta_1^{hi}$ population was selected as previously described (Collins et al., 2001). Briefly collagenase (Worthington, UK) digestion of prostatic tissue released epithelial acini and ducts, which were separated from the stromal fraction by differential gravity centrifugation. This method yields epithelial purity of >98% (Robinson et al., 1998). Subsequently, the acini were treated with trypsin/EDTA (Life Technologies, Paisley, UK) for 30 minutes at 37°C to release a single-cell suspension. To further purify this population, immunomagnetic depletion was used to remove luminal cells using CD57 antibody (Serotec, UK) (Liu et al., 1997) and a secondary IgM antibody conjugated to magnetic beads (Dynal Biotech, UK). The remaining basal cells were selected for human epithelial antigen (HEA) using the MACS HEA cell isolation kit (Miltenyi Biotec, Surrey, UK). To enrich for the stem-cell population, basal cells expressing high levels of integrin $\alpha_2\beta_1$ were selected by rapid adherence to type I collagen (5 minutes) (BD Biosciences, UK). The isolated cells were maintained in keratinocyte medium (KSFM) (Invitrogen, UK) supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml) in the absence of androgen (serum-free medium was used to isolate the effects of KGF treatment on differentiation). From a typical 2-4 g tissue sample approximately 10^4 $\alpha_2\beta_1^{hi}$ cells were isolated.

The $CD133^+$ cells were isolated from the $\alpha_2\beta_1^{hi}$ basal-cell population using MACS microbeads linked to antibody AC133 (Miltenyi Biotec, Surrey, UK) (Richardson et al., 2004). In brief, cells were incubated with 100 μl MACS microbeads linked to antibody AC133, 100 μl FcR blocking buffer, 300 μl MACS buffer, and then incubated at 4°C for 1 hour. After incubation, cells were washed twice in MACS buffer followed by centrifugation and run through a magnetic mini-MACS column. The column was washed with a further 1.5 ml MACS buffer and the cells eluted from the column constituted the $CD133^-$ cell population. The $CD133^+$ cells were subsequently flushed from the MACS column in 1 ml MACS buffer.

Antibodies

For the β_1-blocking experiments, a neutralising antibody was used (3S3 Serotec, UK; 80 μg/ml). After washing the treated cells, epithelial differentiation of prostatic cells was validated using antibodies specific for PAP (rabbit A0627, Dako, UK), PSA (goat sc-26023, Santa Cruz Biotechnologies), CK18 (mouse F7212, Dako) and AR (rabbit sc-815, Santa Cruz Biotechnologies). To avoid the potential risk of non-specific binding of the secondary conjugates with the mouse monoclonal β_1-blocking antibody, antibodies used to assay against the markers of differentiation were raised in non-mouse species or directly conjugated. Expression of these differentiation markers were assessed by immunofluorescence with confocal microscopy and FACS analysis. Furthermore, the effect on the expression of the $\alpha_2\beta_1$ integrin was examined (anti-$\alpha_2\beta_1$, PIE6, Dako). Cells with no primary antibody and an isotype IgG were used as controls.

Methylcellulose suspension

To induce differentiation, cells were suspended in methylcellulose (MC) as described by Green (Green, 1977). Briefly, cells were suspended in 1% MC (Sigma, UK) for 1 hour and then washed and re-plated onto collagen type-1 for 24 hours. Differentiation was subsequently assessed by expression of the differentiation-specific marker, PAP by immunofluorescence microscopy. This experiment was repeated in triplicate.

Effect of β_1-blocking antibody and KGF on $\alpha 2\beta 1^{hi}$ epithelial cell differentiation

Having examined the effect of cell suspension on differentiation, the more specific effects of blocking the $\alpha_2\beta_1$ integrin and the role of KGF was explored. The $\alpha_2\beta_1$ integrin bright cells were treated for 1 hour with β_1-blocking antibody and/or KGF (10 nM). The cells were subsequently washed with PBS and prepared for FACS analysis or re-plated. The cells that were re-plated were left to adhere for 24 hours to allow maximal capture of cell number, as blocking the β_1 integrin reduces adherence (see Results), prior to fixing on collagen-1 plates for immunofluorescence examination. To study the effects on adhesion, the selected cells were incubated with β_1-blocking antibody for 1 hour and re-plated onto collagen-1 slides. Cells were washed with PBS to remove non-adherent cells. Cell adhesion was measured by enumeration using 1.6% Trypan Blue (Gibco, UK) exclusion to determine cell viability. For the adhesion assay, at least 2000 cells per experiment arm were counted and these experiments were repeated in triplicate.

Immunofluorescence of treated $\alpha_2\beta_1^{hi}$ cells

The cells were prepared for double- or triple-labelled immunofluorescence. Following treatment with appropriate experimental conditions, cells on the collagen-1-coated tissue-culture slides (BD Biosciences, Oxford, UK) were fixed in methanol at –20°C and air dried. Subsequently, the fixed cells were permeabilised using 0.5% Triton-X100 (Sigma, UK) before blocking with 20% blocking serum and incubated with labelled antibodies. Cell nuclei were subsequently stained with Diamino-2-phenylindole (DAPI, Vectashield, UK) and the slides were analysed by confocal microscopy.

Flow cytometry analysis of the treated $\alpha_2\beta_1^{hi}$ cells

The selected and treated cells were prepared for cell cycle or detection of differentiation markers using FACS analysis. For the detection of epithelial differentiation, the cells were permeabilised with 0.01% Triton-X100 to allow the detection of intracellular antigens. The permeabilised cells were incubated with primary anti-human antibodies for 1 hour at 4°C. After washing in Isoton (Coulter, Luton, UK), samples pre-incubated with unconjugated primary antibody were subsequently incubated with a fluorochrome-conjugated antibody for 30 minutes at 4°C. Just prior to FACS analysis, propidium iodide (PI) at a final concentration of 0.025 μg/ml was added to gate out debris. Data analysis was performed using Lysis II software (Becton Dickinson). Increases in expression of the markers of differentiation were taken over 3% of isotype control on quadrant analysis.

Cell cycle analysis following treatment of the prostatic epithelial $\alpha_2\beta_1^{hi}$ cells with the β_1-blocking antibody was performed using flow cytometry. The treated cells were incubated in 0.025 μg/ml PI and 0.025% Triton-X for 15 minutes on ice before flow cytometry to measure DNA content in FL2. To discriminate against nuclear debris and doublets, the cells were gated on FSS, SSC and FL2-W. Flow cytometry cell cycle software (Multicycle for Windows) was used to calculate DNA content and the Watson-pragmatic model was applied for final cell cycle analysis. The assay was repeated in triplicate.

SEM

The epithelial cells, following treatment, were washed and fixed in 2% glutaraldehyde for 1 hour before washing and dehydration through a graded series of ethanol solutions (10-100%). The cells were dried from CO_2 in a critical-point dryer and gold plated for analysis with a scanning electron microscope.

Reverse transcription PCR (RT-PCR) for KGFR

TRIzol (Gibco BRL, UK) reagent was used for the isolation of total RNA from the selected cells. RT-PCR was performed using primers 5′-ATT GTT CTC CTG TGT CTG-3′ (forward) and 5′-CTT TTC AGC TTC TAT ATC C-3′ (reverse) to examine for KGFR (FGFIIIb; GenBank accession number m80637) mRNA expression (Mehta et al., 2000). The PCR thermo-cycling parameters were 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 30 seconds.

Statistics

The paired *t*-test was applied to examine for statistical significant changes between treatment groups. The statistical software package Arcus Quickstat Biomedical Version 1.1 (Research Solutions, UK) was used to analyse the data. The threshold for significance was taken as $P<0.05$.

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