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Research Article

The Role of the Glucocorticoid System in Anchorage-independence during Progression of Squamous Cell Carcinoma

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Abstract

Background. Topical and systemic corticosteroids are widely used for the treatment of a variety of mucocutaneous disorders including those with malignant potential. We have shown previously that normal keratinocytes regulate the local concentration of active steroids, as well as synthesize hydrocortisone de novo following stimulation with ACTH. The role of locally produced hydrocortisone in tumour progression is unclear.

Objective. To examine the ability of endogenously-produced and exogenously-administered hydrocortisone on the capacity of human keratinocytes to promote the formation of anchorage-independent multicellular aggregates (MCAs) in vitro.

Methods. The human keratinocyte cell line HaCaT derived from irradiated facial skin and c-Ha-ras-transfected HaCaT cell clones (I-7, II-3, RT-3) demonstrate increasing tumorigenic and metastatic potential in vivo and are ideally suited to studies of keratinocyte tumour progression. The formation of multicellular aggregates (MCAs) in vitro was used as a paradigm of metastatic potential in vivo. Cortisol levels were assessed by ELISA.

Results. Non-tumorigenic (HaCaT), benign (I-7), malignant with low metastatic potential (II-3) and malignant with high metastatic capacity (RT-3) keratinocytes secreted basal levels of cortisol, activated cortisone and differentially synthesized cortisol de novo in the presence of ACTH; malignant cells (II-3 and RT-3) produced the highest levels of the steroid. When the cells were cultured in the absence of a substrate,

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RT-3 cell in particular were highly effective in forming MCAs and hydrocortisone further promoted MCA formation. Stimulation of the endogenous glucocorticoid system by ACTH caused an increase in MCA formation by II-3.

Conclusion. The data demonstrate that stimulation of the endogenous glucocorticoid system promotes anchorage independence of malignant keratinocytes in vitro. The results question the use of corticosteroids as therapeutic agents for potentially malignant conditions.

Keywords: Squamous cell carcinoma; Epidermal glucocorticoid system; Keratinocytes; Multi-cellular aggregates; Glucocorticosteroids; Metastatic phenotype

1. Introduction

Glucocorticoids are among the drugs most widely used in Oral Medicine and Dermatology. Topical and systemic preparations of corticosteroids are the mainstay for the treatment of a large number of diseases that require anti-inflammatory activity and immunosuppression (Hengge et al., 2006; Jackson et al., 2007), including potentially malignant conditions such as Lichen Planus (Popovsky and Camisa, 2000).

Glucocorticoid hormones such as hydrocortisone are produced in the adrenal cortex and exert pleiotropic effects in peripheral tissues by regulating the expression of up to 10% of genes associated with broad spectrum of metabolic processes (John et al., 2009). In addition to the adrenal-derived steroids, it is now recognised that peripheral tissues may also act as steroidogenic organs. The epidermis, for example, exhibits powerful metabolic and endocrine capabilities and is now regarded as part of a peripheral HPA axis (Slominski and Wortsman, 2000; Slominski et al., 2007; 2013). We have shown, for example, that oral keratinocytes regulate the local concentration of active steroids as well as synthesize hydrocortisone de novo following stimulation with ACTH (Cirillo and Prime, 2011) and previous reports have demonstrated that corticosteroidogenesis can proceed from cholesterol (Slominski et al., 2004). Further, recent data suggest that deregulation of the endogenous glucocorticoid system in keratinocytes may be involved in epithelial malignancy (Cirillo et al., 2012).

The relationship between inflammation and cancer has been under intense scrutiny in recent years (Erdman et al., 2010; Grange et al., 2011). Certain non-adrenal cell types, including cancer cells, are able to endogenously produce molecules such as hydrocortisone that have anti-inflammatory properties (Cirillo and Prime, 2011; Sidler et al., 2011), but the mechanism by which these molecules regulate tumour growth are unclear. Tumour-derived corticosteroids may suppress the immune response to favour cancer cell escape from T cells and NK cells as demonstrated in colon cancer (Sidler et al., 2011) or alternatively, may enhance cancer cell metabolism as shown in glioblastoma (Seyfried et al., 2010). Whether the metastatic behaviour of cancer cells is influenced specifically by hydrocortisone is not known.

Squamous cell carcinoma (SCC) is one of the most common cancer types in man and by far the most common epithelial malignancy in the oral cavity (Hillbertz et al., 2012). SCC of the head and neck (HNSCC) is the sixth most prevalent neoplasm in the world with approximately 900,000 cases diagnosed annually worldwide and a 5 year mortality rate of 50% (Chin et al., 2006). The disease has reached epidemic proportions in India and South East Asia and disturbingly, the incidence is

increasing in the West (Conway et al., 2006). Local invasion and metastases to regional lymph nodes are the primary causes of death (Parkin et al., 2001). A key mechanism enabling cancer cells to invade and metastasize is their ability to aggregate and survive in the absence of attachment to an extracellular matrix (ECM). This occurs primarily when the tumour cells float freely in the blood and lymphatic vessels where they escape anoikis to form multicellular aggregates that facilitate anchorage independent survival (Zhang et al., 2004).

In the present study, we examined whether endogenous or exogenously-administered hydrocortisone in human keratinocytes promoted the formation of anchorage independent 3D aggregates in vitro. We used the HaCaT cell line derived from irradiated human facial skin and c-Ha-ras-transfected HaCaT cell clones (I-7, II-3, RT-3) because the cells share a common genetic background, individual lines demonstrate increasing tumorigenic and metastatic potential in vivo and the cells have been used extensively by the scientific community as a model of keratinocyte tumour progression (Boukamp et al., 1988; Prime et al., 1990; Fusenig et al., 1998). The results show for the first time that hydrocortisone promotes the anchorage independent phenotype which is recognized as being pivotal for the metastatic dissemination of tumour cells.

2. Materials and Methods

2.1 Cell culture and reagents

Details of the spontaneously immortalised human skin keratinocyte cell line (HaCaT) and the mutant c-Ha-ras-transfected HaCaT cell clones (I-7, II-3, RT-3) have been reported previously (Fusening and Boukamp, 1998). Following subcutaneous transplantation to athymic mice, HaCaT cells are non-tumorigenic, I-7 cells form non-invasive epidermoid cysts, II-3 cells form primary SCCs with minimal metastatic dissemination and RT-3 cells, the most aggressive cell type, form large SCCs at the site of inoculation and widespread metastatic dissemination (Boukamp et al., 1990; Fusening and Boukamp, 1998). Cells were grown without mesenchymal support in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) in a humidified atmosphere of 5% CO2/air at 37 °C. Ha-ras-transfected clones (I-7, II-3, RT3) were grown in media containing 400 g/ml geneticin sulphate (G418; PAA laboratories, UK). All other reagents for cell culture were from Sigma (Sigma-Aldrich, Gillingham, Dorset, UK).

In experiments involving cortisol measurements, cells were incubated in the absence and presence of cortisone (100nM) and ACTH (10nM) in serum-free DMEM whilst the quantification of MCAs was undertaken in the absence and presence of cortisone (100nM), hydrocortisone (100nM) and ACTH (10nM).

2.2 Preparation of multicellular aggregates (MCAs)

Multicellular aggregates (MCAs) were prepared as described previously (Kantak and Kramer (1998), with minor modifications. Briefly, keratinocyte monolayers were treated with trypsin-EDTA to prepare single cell suspensions which were then plated on polyhydroxylethyl-methacrylate (poly-HEMA)-coated 60-mm dishes (6 x 105 cells/dish) in the presence of serum-free DMEM. The number of spheroids formed was calculated by 1) Counting MCAs in ten representative fields ± standard deviation; 2) Counting the total number of cells which formed spheroids. This

latter measurement was determined by collecting MCAs from individual culture dishes, transferring to 15 ml tubes and centrifuging for 5 min at 1g. In these conditions, only aggregates sedimented whilst single cells remained in suspension. Cells were counted using a hemocytometer or by manual counting. The rate of MCA-forming cells was obtained indirectly by counting cells in suspension or directly by counting MCA-sedimented cells following trypsinization.

2.3 Trypan Blue Exclusion Test

The Trypan Blue Exclusion test was performed using standard procedures (Strober, 2001) in order to determine the number of viable cells in the MCAs; viable cells had a clear cytoplasm whilst non-viable cells absorbed dye resulting in a blue cytoplasm.

2.4 Assessment of cortisol levels by ELISA

Keratinocytes were grown to confluence in 35-mm Petri dishes and conditioned media collected after 24 hours. Cortisol levels were assessed with a Cortisol Parameter Assay Kit (R&D System) and quantified at 415 nm using the ELx808 microplate reader (BioTek Instruments, Inc., Winooski, VT). Absence of cortisol in the control serum-free media was also checked by ELISA.

2.5 Statistical analysis

The statistical significance of the data was evaluated by the Student's t test. Data are reported as mean ± standard deviation (SD) and differences were considered to be significant with $p<0.05$.

3. Results

3.1 Malignant HaCaT clones secreted higher levels of endogenous hydrocortisone in response to ACTH compared to non-malignant clones.

Previous studies demonstrated that non-tumorigenic HaCaT cells produced basal levels of cortisol which was increased by ACTH (Cirillo and Prime, 2011). In the present study, we investigated whether a functioning glucocorticoid system was active in cancer cells. We show that all of the cell lines secreted basal levels of cortisol as measured by ELISA, were able to convert inactive cortisone to active cortisol and synthesized significantly higher levels of cortisol de novo following stimulation with ACTH compared to untreated controls; cortisol neogenesis after ACTH treatment was significantly more pronounced in the malignant clones (II-3, RT-3) than the non-malignant cell lines (HaCaT, I-7) (Figure 1). The data demonstrate that the epidermal glucocorticoid system is active in cancer cells and that the production of cortisol is associated with progression in epithelial malignancy.

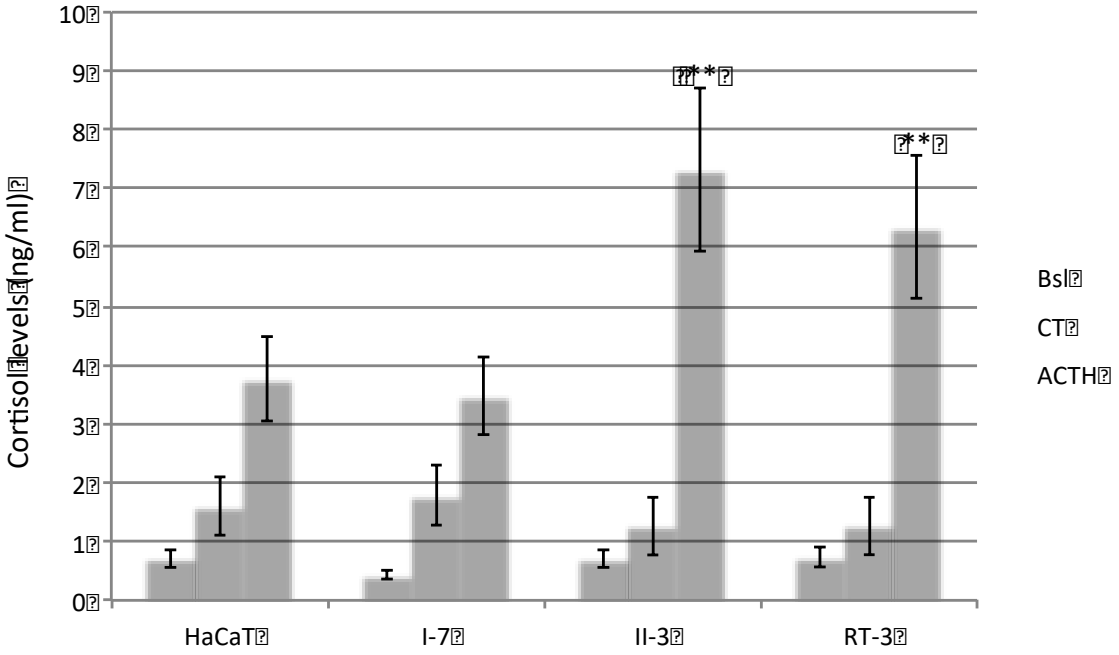

Fig. 1. Cortisol levels were assessed by ELISA in the HaCaT series (HaCaT, I-7, II-3, RT-3) under basal conditions (Bsl) and after treatment with 100nM cortisone (CT) and 10nM ACTH. II-3 and RT-3 displayed a significantly (*, $p<0.05$) higher production of cortisol upon stimulation with ACTH, compared with HaCaT.

3.2 Formation of MCAs and anchorage-independent survival

Whilst the ras-transfected HaCaT clones show increasing tumorigenic and metastatic potential when transplanted in vivo, there are only a limited number of parameters that reflect their biological behaviour in vitro. We demonstrate that when the HaCaT series of cell lines were cultured with a non-adhesive substrate (poly-HEMA), only RT-3 cells were able to form significantly more MCAs than their non-malignant (HaCaT, I-7) and malignant (II-3) counterparts (Figure 2). The results support the view that malignant keratinocytes which have the capacity to metastasize widely in vivo have acquired the potential for anchorage independent growth in vitro and support the concept that MCA formation is a useful in vitro hallmark of advanced epithelial cancer.

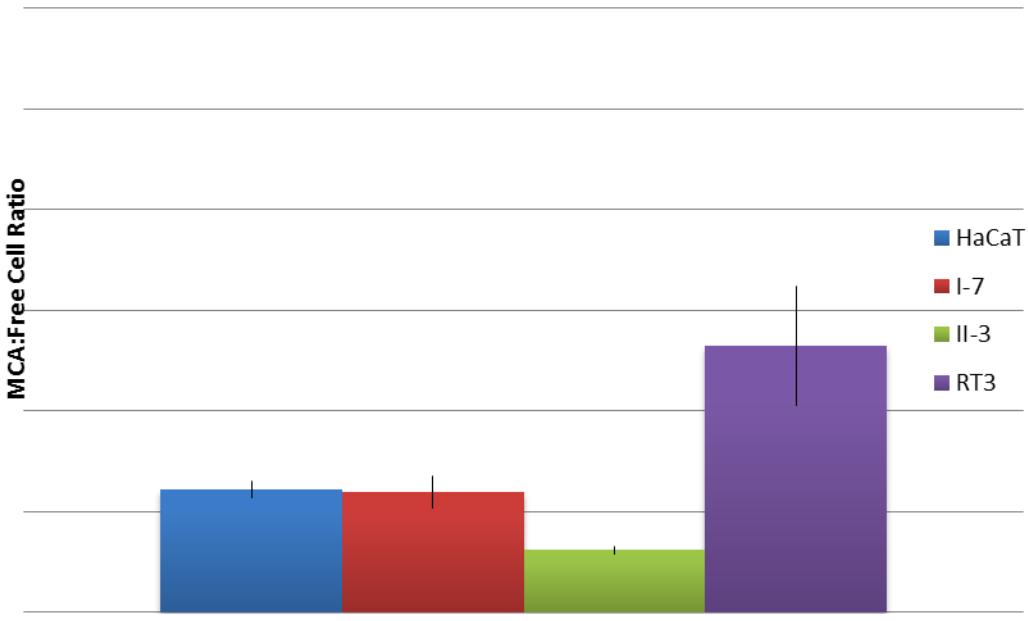

Fig. 2. The cells were trypsinized and quenched before being aliquoted into the poly-HEMA coated wells. The cells were then incubated at 37 °C 5%C02 for 24 hours to allow time for the cells to form MCAs. After 24 hours, the cells were removed from the wells and separated based on their sedimentation-cells that formed MCAs sedmiented to the bottom while cells that had remained free would remain in solution. The cells were then trypsinzed in order to ensure that they were in single cell formation where they could be counted using a Coulter counter. A ratio was worked out for MCA: Free cells and the results are graphed taking the mean of the three repeats with error bars shown for standard deviation. RT-3 displayed a significantly ($p<0.01$) higher rate of MCA formation compared with HaCaT.

3.4 Hydrocortisone enhanced MCA formation in malignant cells

We tested the ability of endogenous and exogenous hydrocortisone to promote the formation of mature spheroids (Table 1). Non-malignant cells (HaCaT, I-7) displayed a low tendency to form MCAs following steroid stimulation, as shown by the MCA:free cell ratio (Figure 3 A,B). II-3 exhibited a relatively poor ability to form spheroids in normal conditions but both ACTH and hydrocortisone enhanced the MCA:free cell ratio in these cells (Figure 3C); the majority of II-3 spheroid-forming cells treated with ACTH were non-vital (Supplementary Figure 1). By contrast, RT-3 was highly effective in aggregate formation in the absence of ECM attachment and this characteristic was further enhanced in the presence of hydrocortisone (Figure 3D). Similar results were obtained when the number of vital cells forming spheroids were counted (Supplementary Figure 2). The results demonstrate that the anchorage independent phenotype in malignant keratinocytes may be enhanced by hydrocortisone.

Table 1 Effect of cortisone, hydrocortisone and ACTH, and absence of treatment (control), on the ability of HaCaT and c-Ha-ras-transfected HaCaT cell clones (I-7, II-3, RT-3) to grow in the absence of substrate. ANOVA statistical analysis was used to determine statistical significance.. Mean values of four independent experiments ± standard deviation (%) are reported. §, P<0.05 RT-3 vs. HaCaT; *, P<0.05 vs. Control

	HaCaT	I-7	II-3	RT-3
Control	1.22 ± 0.08 (6.80%)	1.19 ± 0.16 (13.38%)	0.62 ± 0.03 (6.01%)	2.65 ± 0.60 (22.44%)§
Cortisone	0.97 ± 0.44 (45.34%)	1.05 ± 0.15 (14.45%)	0.57± 0.12 (20.48%)	2.17 ± 1.11 (51.23%)
Hydrocortisone	1.13 ± 0.36 (31.93%)	1.28 ± 0.89 (69.19%)	0.84 ± 0.07 (8.43%)*	5.41 ± 0.41 (7.57%) *
ACTH	0.66 ± 0.12 (17.67%)	1.02 ± 0.54 (53.03%)	1.816±0.99 (54.51%)	1.84 ± 0.79 (42.88%)

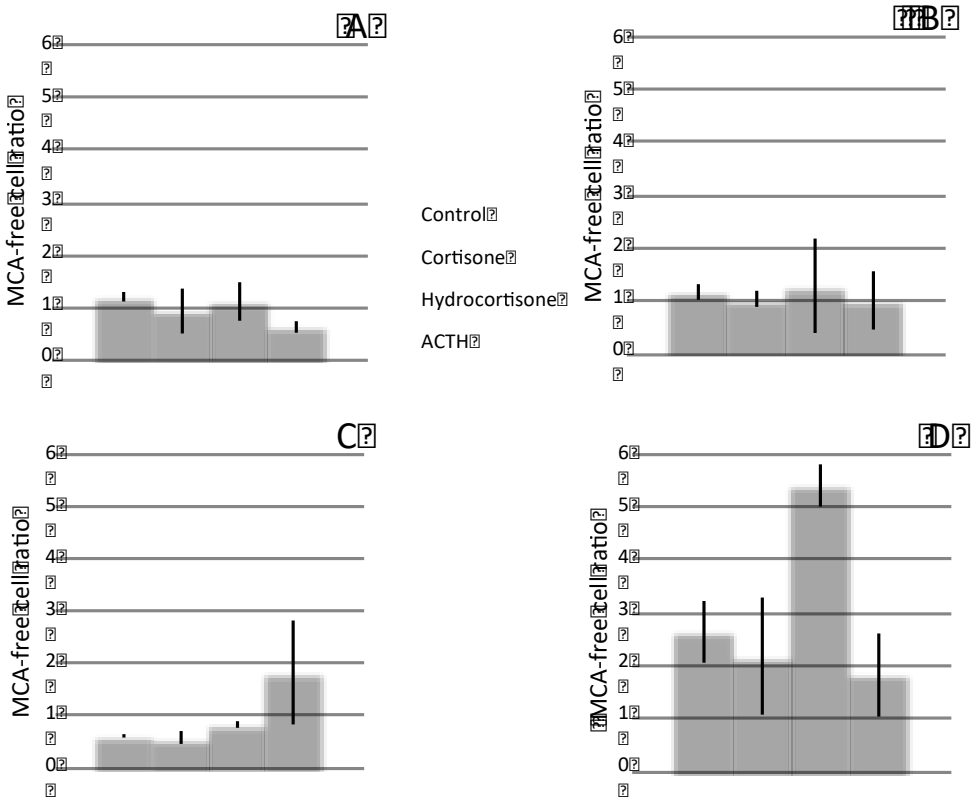

Fig. 3. HaCaT (A), I-7 (B), II-3 (C), and RT-3 (D) were seeded into poly-HEMA coated wells to allow for MCA formation in the presence of a test compound (100nM cortisone, 100nM hydrocortisone, 10nM ACTH) or without treatment (Control). ANOVA statistical analysis was used to determine significance of results. The histograms show mean values of four independent experiments± standard deviation. Refer to table I for statistical significance.

4. Discussion

In the present study, we explored the interaction between glucocorticoids and anchorage independence in vitro in a model of epithelial cancer progression. Specifically, our data show for the first time that both benign (HaCaT, I-7) and malignant (II-3, RT-3) keratinocytes synthesized cortisol, with the levels of cortisol being elevated across all cell lines following treatment with ACTH but particularly in the malignant cell lines. Previous studies have shown that hydrocortisone may act synergistically with adrenaline and noradrenaline to stimulate production of MMP-9 (Thaker et al., 2007) and this may account for the increased invasiveness of ovarian cancer cells when treated with cortisol (Sood et al., 2006).

To evaluate the capacity of keratinocytes to grow in the absence of a substrate, cells were grown on plates coated with poly-HEMA which is known to simulate cell growth in the absence of ECM attachment. In these circumstances, normal cells undergo anoikis whereas cancer cells aggregate together and avoid anoikis through suppression of p53-mediated signals (Zhang et al., 2004). In the present study, all of the cell lines formed MCAs but the highly metastatic lines (RT-3) formed significantly more than the weakly metastatic (II-3), non-tumorigenic (HaCaT) or benign (I-7) keratinocytes. The results support the widely held view that metastatic cells escape the need for anchorage dependent cell growth (Freedman and Shin, 1974; Gassmann and Haier, 2008).

To determine the effect of the different components of the glucocorticoid pathway on the ability of cells to aggregate and survive without matrix attachment, the HaCaT series of lines were treated with cortisone, hydrocortisone and ACTH. We show that hydrocortisone increased MCA formation in RT-3 cells by some 200%, findings that suggest that hydrocortisone may confer a metastatic advantage in vivo. Kodama and Kodama (1975) were the first to show that increased levels of hydrocortisone increased tumour metastasis but the mechanisms that underlie this phenomenon are unclear. Hydrocortisone influences a broad spectrum of keratinocyte behavior including proliferation, differentiation, inflammation and apoptosis (Hannen et al., 2010). With respect to the role of hydrocortisone in tumour metastasis, unfortunately the majority of studies have been confined to breast cancer. In early stage disease, hydrocortisone levels are low, but as the breast cancer becomes more aggressive and starts to metastasize, hydrocortisone levels increase. Further, in healthy people, hydrocortisone levels tend to peak in the morning whereas in breast cancer patients, the circadian rhythm is disturbed and there are multiple peaks either throughout or towards the end of the day (Palesh et al., 2008). Dysregulation of the HPA axis is likely to account for these abnormal rhythms but, at present, it is not clear what the impact of these anomalies are in the skin where the physiology of keratinocytes differs significantly from breast epithelial cells.

Tumours formed by RT-3 cells are poorly differentiated and highly aggressive. Interestingly, RT-3 cells also express constitutively Granulocyte Colony-Stimulating Factor (G-CSF) and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) (Fusening and Boukamp, 1998). Furthermore, the expression of G-CSF and GM-CSF has been detected in a number of different solid tumours, including the tongue (Horii et al., 1997), where it is thought that they are crucial to tumour growth and invasion (Mueller et al., 1999) and act as potent activators of angiogenesis. In keratinocytes, G-CSF and GM-CSF are only expressed during wound healing where the cytokines stimulate proliferation and migration. The constitutive expression of G-CSF and GM-CSF by highly malignant keratinocytes, therefore, most probably reflects a shift from paracrine to autocrine growth

regulation with independence of control by the surrounding stroma (Obermueller et al., 2004). Interestingly, G-CSF and GM-CSF are regulated by hydrocortisone in a cell type-specific manner (Rinehart et al., 1997).

In the present study, the addition of ACTH to II-3 cells increased the ratio of MCA:free cells. We have shown recently that keratinocytes respond to exogenous ACTH by synthesizing hydrocortisone de novo and the II-3 cell line specifically over-expresses HSD11β which converts inactive cortisone to hydrocortisone (Cirillo and Prime, 2011). It is not clear, however, that the increased levels of hydrocortisone in II-3 cells provide a metastatic advantage in these cells because the Trypan Blue Exclusion Test showed that most of the cells that formed MCAs under these conditions were non-viable. Interestingly, HSD11β1 expression is also moderately up regulated in the I-7 cell line (Cirillo and Prime, 2011) but the results of the present study showed no evidence of an increase in the MCA:free cell ratio following treatment with ACTH in this cell line.

In theory, the addition of cortisone to the HaCaT series of lines which over-express the bi-directional HSD11β1 enzyme should lead to increased synthesis of hydrocortisone because the cells have an increased amount of cortisone from which they can synthesize cortisol. The results of the present study, however, do not support this hypothesis because the MCA:free cell ratio remained unchanged despite the addition of cortisone. It may be that the levels of HSD11β1 were insufficient to stimulate conversion of cortisone to hydrocortisone or, alternatively, the rate of de-activation by the HSD11β2 isoform prevailed over the increased rate of activation by HSD11β1.

In conclusion, this study highlights key aspects of the interaction between glucocorticoids and keratinocytes during cancer progression. Non-tumorigenic (HaCaT) and benign (I-7) keratinocytes did not form MCAs to any significant degree even after the addition of hydrocortisone. By contrast, malignant keratinocytes with low levels of metastases (II-3) showed comparable levels of MCA formation as HaCaT and I-7 cells but MCA formation was stimulated following treatment with ACTH. The most aggressive cell type that is characterized by widespread metastases in vivo (RT-3) demonstrated relatively high levels of MCA formation which was further enhanced by the addition of hydrocortisone. Taken together, the data suggest that the epidermal glucocorticoid system is associated with tumour progression and that cortisol plays a key role in metastatic dissemination. It is suggested that this phenomenon merits further investigation particularly in light of the widespread use of synthetic corticosteroids in the management of potentially malignant conditions.

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Conflict of Interest

None

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