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Characterisation of the cancer-associated glucocorticoid system: key role of 11β-hydroxysteroid dehydrogenase type 2

By

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Running Title: Glucocorticoid system in malignancy

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ABSTRACT

Background. Recent studies have shown that production of cortisol not only takes place in several non-adrenal peripheral tissues such as epithelial cells but, also, the local inter-conversion between cortisone and cortisol is regulated by the 11β-hydroxysteroid dehydrogenases (11β-HSDs). However, little is known about the activity of this non-adrenal glucocorticoid system in cancers.

Methods. The presence of a functioning glucocorticoid system was assessed in human skin squamous cell carcinoma (SCC) and melanoma and further, in 16 epithelial cell lines from 8 different tissue types using ELISA, Western blotting and immunofluorescence. 11β-HSD2 was inhibited both pharmacologically and by siRNA technology. Naïve CD8+ T cells were used to test the paracrine effects of cancer-derived cortisol on the immune system *in vitro*. Functional assays included cell-cell adhesion and cohesion in two- and three-dimensional models. Immunohistochemical data of 11β-HSD expression were generated using tissue microarrays of 40 cases of human squamous cell carcinomas as well as a database featuring 315 cancer cases from 15 different tissues.

Results. We show that cortisol production is a common feature of malignant cells and has paracrine functions. Cortisol production correlated with the magnitude of glucocorticoid receptor (GR)-dependent inhibition of tumour-specific CD8+ T cells *in vitro*. 11β-HSDs, were detectable in human skin SCCs and melanoma. Analyses of publicly available protein expression data of 11β-HSDs demonstrated that 11β-HSD1 and -HSD2 were dysregulated in the majority (73%), of malignancies. Pharmacological manipulation of 11β-HSD2 activity by 18βGlycyrrhetinic Acid (GA) and silencing by specific siRNAs modulated the bioavailability of cortisol. Cortisol also acted in an autocrine manner and promoted cell invasion *in vitro* and cell-cell adhesion and cohesion in two- and three-dimensional models. Immunohistochemical analyses using tissue microarrays showed that expression of 11β-HSD2 was significantly reduced in human SCCs of the skin.

Conclusion. The results demonstrate evidence of a cancer-associated glucocorticoid system and show for the first time, the functional significance of cancer-derived cortisol in tumour progression.

Keywords: cancer, cortisol, lymphocytes, 11β-HSDs, keratinocytes, cell adhesion

Introduction

Glucocorticoids (GC) are important lipid hormones that are involved in the regulation of stress responses, metabolism and immune homeostasis (Sapolsky *et al*., 2000). Whilst the adrenal glands are the major source of GC, it is now recognized that GC synthesis occurs at other sites (Noti *et al*., 2009) such as the thymus (Vacchio *et al*., 1994), brain (MacKenzie *et al*., 2000; Davies & MacKenzie, 2003), prostate gland (Herr and Pfitzenmaier, 2006), vascular endothelium (Takeda *et al*., 1994), intestinal epithelium (Sidler *et al*., 2011) and epidermis (Slominski & Wortsman, 2000; Hannen *et al*., 2011). In the skin, steroidogenesis is involved in the modulation of a variety of physiological functions (Slominski *et al*., 2000; Vukelic *et al*., 2011; Kennedy *et al*., 2015). Recently, we characterized the oral glucocorticoid system in detail and showed that the local concentration of active cortisol in normal keratinocytes had the capacity to modulate disease progression in conditions where steroids are used routinely as a treatment modality (Cirillo & Prime, 2011; Cirillo *et al*., 2012). These findings raise the possibility that steroidogenesis may also play a key role in carcinogenesis, possibly through autocrine and paracrine mechanisms. Given the known immunomodulatory function of cortisol, we wished to examine whether local non-adrenal steroids had the capacity to modulate anti-cancer immune responses.

Regulation of neuroendocrine function occurs primarily through the control of the production and degradation of steroid hormones (Slominski & Wortsman, 2000). Activation of the steroid cortisone to cortisol occurs via 11β-hydroxysteroid dehydrogenase (HSD) 1 (*HSD11B1* gene; Tomlinson *et al*., 2004) whereas the reverse, cortisol to cortisone, involves 11β-HSD2 (*HSD11B2* gene; Ma *et al*., 2011). 11β-HSDs, therefore, are key enzymes in the tissue-specific regulation of glucocorticoids and current

thinking suggests that their deregulation is associated with a variety of pathological processes in the skin (Vukelic *et al*., 2011; Hannen *et al*., 2011, Cirillo *et al*., 2012). Whilst the role of 11β-HSD1 is well documented in epidermal physiology (Terao *et al*., 2011; Tiganescu *et al*., 2011; Lee *et al.,* 2013; Itoi *et al.,* 2013; Terao *et al.,* 2014; Tiganescu *et al*., 2015), little is known about the role of 11β-HSD2 in skin keratinocytes. Clarification of the function of 11β-HSD2 in epidermal keratinocytes may have major clinical implications because of the therapeutic relevance of corticosteroid agents in the management of many skin disorders (Jackson *et al*. 2007).

In the present study, we assessed cortisol production in a broad spectrum of cell and tissue types. We show that cortisol levels correlated with inhibition of proliferation amongst tumour-specific CD8+ T lymphocytes. Using immunohistochemical analysis of cancer samples, we demonstrated alterations of 11β-HSD enzymes in a large number of tissue types, including skin SCC. The role of 11β-HSD2 in normal and malignant keratinocytes was also investigated using functional assays of metabolism, adhesion and scattering from three-dimensional aggregates. The data demonstrate for the first time that 11β-HSD2 plays a key role in the pathophysiology of malignant epidermal cells.

Materials and methods

Antibodies and chemicals

Antibodies against residues 261-405 of human 11β-HSD2 (clone H-145), and amino acids 65-164 of 11β-HSD1 (clone H-100), anti-tubulin, horseradish peroxidase (HRP)-

conjugated and FITC-conjugated anti-rabbit IgGs and 18β-Glycyrrhetinic acid (18β-GA) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nitrocellulose membranes, keratinocyte growth medium and antibiotics/anti-mycotics were obtained from Invitrogen (Paisley, UK). Reagents for enhanced chemiluminescence and films were obtained from Amersham Biosciences (Buckinghamshire, UK) and the reagents for protein extraction and cell culture were obtained from Sigma (Sigma-Aldrich, Gillingham, Dorset, UK).

Cells and culture conditions

A broad spectrum (n=16), of normal and malignant cell lines from 8 different tissue types were used in this study including prostate (DU145, PC3, VCaP, LNCaP), bladder (T24, RT4), breast (MCF-7), colorectal (HT29, SW620, SWH80), renal (mouse RenCA WT) and pancreas (HUp-T3; a kind gift from Professor Chris Paraskeva, School of Cellular and Molecular Medicine, University of Bristol, UK). These cells were validated using karyotypic analysis prior to use. These cells retained their phenotype and function, together with their growth characteristics *in vitro*, throughout the 3 months that they were routinely kept in continuous culture. HaCaT cells are a strain of immortalized human skin keratinocytes that have an active steroid metabolism (Cirillo and Prime, 2011) and display normal differentiation *in vivo* (Boukamp *et al*., 1988; Milewich *et al*., 1988). HaCaT c-Ha-ras-transfected clones (I-7, II-3, and RT-3; Fusenig and Boukamp, 1998) were kind gifts from Professor Norbert Fusenig (DKFZ, Heidelberg, Germany). 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 have widespread metastatic dissemination (Fusening and Boukamp, 1998). The cells are widely used as a model of epidermal cancer progression (Davies et al., 2006) and were last authenticated prior to commencing the experiments in 2012 with mRNA microarrays. The oral SCC cell strain (H357) underwent full genome sequencing in 2016 whereas the skin SCC cell line A431 was purchased from Sigma Aldrich.

All of the cell lines/strains were derived prior to 2001 and therefore, were not subject to Ethical Committee approval in the UK. The cells were placed in serum-free DMEM/F12 for 24 hours before the collection of supernatants for cortisol assessment; RT-3 was cultured in media containing 400mg/ml geneticin sulphate (G418; PAA laboratories, UK). At the time of experimentation, cells were seeded in 35-mm Petri plastic dishes and grown to confluence. Cells were grown in a humidified atmosphere of 5% CO_2/air at 37°C. Ethics committee approvals were was obtained for this study both in Bristol (E5133) and in Melbourne (1340716.1), and high standards of ethics was applied in carrying out all of the investigations.

Multicellular aggregates (MCAs) were prepared according to published protocols (Kantak and Kramer, 1998). To generate MCAs, cell monolayers were first treated with EDTA to prepare single cell suspensions and then, the cells were seeded onto polyhydroxylethyl-methacrylate (poly-HEMA)-coated 60-mm dishes (6×10^5 cells/dish) in the presence of serum-free DMEM for 12 hours, with or without 0.5% (w/v) 18β-Glycyrrhetinic Acid (GA). Unlike malignant keratinocytes (Kantak & Kramer, 1998), the culture of normal keratinocytes for prolonged periods (e.g. 24 hours) in the absence of extracellular cell

matrix (ECM) attachment resulted in significant cell death and non-viable MCAs (data not shown).

Tumour specific CD8+ T lymphocyte proliferation in vitro.

Using anti-CD8 MACS midiMACS (Miltenyi Biotec, Bisley, UK), single-cell suspensions of peripheral lymph node and spleen cells from CL4 TcR–transgenic mice were enriched for naïve $CD8^+$ T lymphocytes that recognized a tumor specific antigen (Jenkinson et al., 2005). Anti-CD3 monoclonal antibody (10µg/ml) was added to triplicate wells of 96-well rounded plates in a total volume of 50µl 1xPBS and incubated in a 5% vol./vol. CO_2 humidified atmosphere at 37^0C for 1.5 hours; control wells contained 1xPBS only. Plates were washed three times with 300µl 1x PBS. In certain experiments, cortisol (10-1000ng/ml) was added to the wells. 5×10^4 purified naïve, CL4, CD8+ T lymphocytes were added to each well and were cultured in a 5% vol./vol. CO_2 humidified atmosphere at 37^0C for 72 hours and pulsed with 1µCi/well ^{3}H-thymidine (Amersham Life Sciences) in 25µl serum-free culture media for the last 8 hours. Cells were harvested on fiber filters (Filtermats; COX Scientific Ltd., Kettering, UK) using a 24-well harvester (Skatron, Flow Laboratories, Oslo, Norway). Fiber filters were then dried and sealed into bags with 3ml Betaplate Scint (Wallac-Oy, (Wallac Oy, Turku, Finland). ^{3}H-thymidine incorporation was read by a 1450 Microbeta liquid scintillation counter for windows 2.7 (Wallac Oy, Turku, Finland). Data were analysed using Microsoft Office Excel and Prism 4.03 software (Graphpad).

siRNA transfection experiments

11β-HSD-directed siRNA pools and the negative-control pool were transfected as described previously (Cirillo *et al*., 2011). The efficiency of transfection was monitored by Western blotting (WB) of 11β-HSD1/2 expression.

Western blotting

For the assessment of protein levels, WB on whole-cell lysates and in-cell WB on culture monolayers was undertaken using standard procedures (Cirillo *et al*., 2010). For all WB, a 1:500 dilution was used for the primary antibodies and the species-specific secondary IgG was diluted to 1:5000.

Enzyme-linked immunosorbent assay (ELISA)

For quantification of cortisol levels with conventional ELISA, the conditioned media were collected at time points specified in the Results. Cortisol concentrations were measured with a Cortisol Parameter Assay Kit (R&D Systems, Minneapolis, MN, USA) and quantified at 415 nm with the ELx808 Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Immunohistochemistry

Skin tissue microarrays (Cat. No. SK483, multiple skin squamous cell carcinoma tissue arrays; US Biomax, Rockville, MD, USA) and formalin-fixed, paraffin-embedded skin biopsies from individuals with squamous cell carcinoma, melanoma, and without skin disease were subjected to immunohistochemical analyses, as described previously (Ricketts *et al*., 1998; Lanza *et al*., 2008). Following antigen retrieval, the primary antibody

to 11β-HSD1 and 11β-HSD2 was used at a 1:100 and 1:50 dilution, respectively, and the species-specific secondary IgG was diluted to 1:5000. The primary antibody was withheld from negative controls and these were incubated in diluent alone. Staining was quantified following the criteria detailed by Yao et al. (2007). Normal and tumour sections were scored independently by two investigators (SSP and NC) and graded as no (0), weak (1), moderate (2), or strong (3) staining.

Database search

A publicly available repository (http://www.proteinatlas.org) was used to access immunohistochemical data of 11β-HSDs staining, as reported in **Supplementary Table I**. GENT (http://medicalgenome.kribb.re.kr/GENT), a Gene Expression across Normal and Tumour tissue database, was used to interrogate mRNA expression in both cell lines and tissues.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as previously described (Cirillo *et al*., 2007), but with minor modifications. Briefly, cells were cultured in 4-well-plates (Nunc™ Cell-Culture Treated Multidishes, Thermo Fisher Scientific, Fisher Scientific UK Ltd**.**), in standard conditions. At 60% confluence, cells were fixed with 100% vol./vol. ice-cold methanol for 10 minutes at 4°C and then incubated with 0.5 μg/ml Hoetstch staining (H6024, Sigma-Aldrich) for 30 minutes at 4°C and then blocked with 5% wt/vol. BSA-PBS solution at 4°C for 60 minutes. Samples were incubated overnight at 4°C with anti-11β-HSD1/2 primary antibody at 1/100 dilution in 2% BSA-PBS followed by FITC-

conjugated species-specific IgG for 1 hour at 1/1000 dilution in 2% wt/vol. BSA-PBS. All intermediate washing steps were performed with PBS. Images were taken with a fluorescence microscope (EVOS™ FLoid™ Cell Imaging Station, Life technologies).

Adhesion and cohesion assays in 2- and 3-dimensional models

To quantify the strength of 2-D cell-to-cell adhesion in monolayers, we undertook dispase-based cell dissociation assays, as described previously (Calautti *et al*., 1998).

For 3-D cohesion assays, MCAs were first analysed by phase-contrast microscopy to check for their compact appearance. Then, the MCAs were sedimented in 15ml tubes and subjected to mechanical stress by pipetting 20 times with a 5ml pipette. The residual spheroids were sedimented at 1G and the ratio of the number of MCA-forming cells and the number of single cells in suspension was calculated. The ratio of total to single cells was indicative of intercellular adhesive strength (Cirillo & Boutros, 2008).

For scattering assays, the surface (pixels) covered by cells originating from disaggregating MCAs were measured and then those values were normalized against the total number of cells.

Statistical analysis

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

Results

Tumour-derived cortisol inhibits lymphocyte proliferation

We and others have shown that tumour-specific $CD8^+$ T lymphocyte activity is suppressed by factors produced within the tumour microenvironment (Ahmadi et al., 2008; Janicki et al., 2008; Basingab et al., 2016). To test whether the production of cortisol by tumour cells had paracrine effects that could target tumour specific immune responses, we exposed naïve tumour-specific $CD8^+$ T lymphocytes (CL4 cells) to conditioned media from cortisol-producing II-3 malignant epidermal cells (Cirillo et al., 2012; Kennedy et al., 2015) stimulated with ACTH and, in parallel, treated lymphocytes with hydrocortisone. The data showed that the rate of proliferation of CL4 $CD8^+$ T lymphocytes was significantly reduced in the presence of conditioned media from ACTH-stimulated II-3 cells; the effect was inhibited in the presence of the GR inhibitor RU486 thereby confirming that such inhibition was specific to cortisol (Figure **1**A). In control experiments we confirmed that hydrocortisone directly inhibited the rate of proliferation amongst naïve mouse tumour-specific CD8+ T cells primed *in vitro* using plate-bound anti-CD3 and anti CD28 mAb in a dose-dependent manner (**Supplementary Figure 1**). We extended these observations to examine whether unstimulated malignant cells from other tissue-types synthesized cortisol *de novo* and, if so, whether conditioned media from these same cells could inhibit naïve tumour-specific CD8+ T lymphocyte proliferation. All (12/12) of the malignant cell lines from the prostate, bladder, breast, colorectum, kidney and pancreas (**Supplementary Figure 2A**), produced detectable levels of cortisol (0.1-8.4 ng/ml) at baseline; 11 of 12 reduced the proliferation rate of CD8+ T lymphocytes and one cell line from bladder cancer (T24) increased the proliferation (**Supplementary Figure 1**). Cell lines of colorectal origin (HT29, SW620, SWH80) produced little, if any, cortisol but

caused marked inhibition of proliferation (**Figure 1B**) suggesting that there may be other mechanisms of immune suppression/regulation exhibited by this cell type (see Discussion). When the data relating to the colorectal lines were excluded, there was a strong correlation between basal cortisol production and inhibition of lymphocyte proliferation (Spearman's correlation coefficient was 0.89; **Figure 1C**). Collectively, the data demonstrate that a large variety of cancer cells from different tissues produce active cortisol and inhibit tumour specific CD8+ T lymphocyte proliferation *in vitro*.

Cortisol interconversion is modulated by 11β-hydroxysteroid dehydrogenases

The above experiments demonstrated that the production of basal levels of cortisol was common in tumour cells. Next, we wanted to test whether the interconversion of cortisol could be modulated locally and, to this end, we used HaCaT skin keratinocytes and their mutant c-Ha-ras-transfected clones (**Figure 1A**). All clones consistently secreted cortisol under basal conditions and increased steroid production following stimulation with ACTH; c-Ha-ras-transfected clones that formed malignant squamous cell carcinomas following transplantation to athymic mice (II-3, RT-3) produced significantly more cortisol following ACTH stimulation than their non-tumorigenic counterparts (HaCaT) and c-Ha-ras-transfected clones that formed benign epidermoid cysts on transplantation to athymic mice (I-7). Other SCC cells derived from tumour samples were also investigated and were found to secrete cortisol under basal conditions (**Supplementary Figure 3**).

Previous studies demonstrated that HaCaT expressed active 11β-HSD1 and 11β-HSD2 enzymes (Cirillo *et al*., 2011, Kennedy *et al*., 2015). In this study, we extended these

observations and demonstrated that benign (I-7) and malignant (II-3, RT-3) c-Ha-ras clones also expressed functional 11β-HSD1 and 11β-HSD2 (**Figure 2B-D**). Silencing of 11β-HSD1 correlated with a reduced ability of malignant cells to convert cortisone to active cortisol; this was progressively restored by an increase of 11β-HSD1 expression (**Figure 2C**). Similarly, 11β-HSD2 controlled the inactivation of cortisol in a dose-dependent fashion (**Figure 2D**). 11β-HSD1 and 11β-HSD2 were also expressed in SCCs (n=10) and melanomas (n=10) (**Figure 2E, F**). Taken together, the data demonstrate that skin cancer cells have the capacity to regulate cortisol levels through the expression of 11β-HSD1 and 11β-HSD2.

Expression of 11β-HSD enzymes is altered in cancers

To investigate whether the enzymes modulating the local concentration of active steroid were dysregulated in malignancy, we examined the expression of 11β-HSD1/2 in normal and malignant tissues using publicly available datasets. According to GENT datasets, mRNA levels encodying for 11β-HSD1 (HSD11B1) and 11β-HSD2 (HSD11B2) differed in normal and neoplastic tissues. Therefore, we examined the expression profile of these enzymes at the protein level using Protein Atlas (http: //www.proteinatlas.org). Fifteen normal tissue types (n = 126; controls) were matched with their malignant counterparts (n = 315; cancers); a total of 441 different samples were examined in which there were 590 cancer specimens (**Supplementary Table I**). 12 of 15 (80%) cancer types had significant changes ($p<0.05$) of 11β-HSDs staining (**Figure 3**). In the main, there was an inverse relationship in the pattern of expression of 11β-HSD1 and 11β-HSD2, with either an increase in 11β-HSD1 staining (cervical, glial, urotelial) or decreased expression of 11β-

HSD2 (colon, lung, stomach, thyroid), or both (endometrial, head and neck, renal, prostate). 11β-HSD1 was also upregulated in liver cancer with a p value close to significance (50% overall increase compared to control tissue, p = 0.069). Exceptions to the above generalisation included breast and pancreatic cancers which did not show major changes compared to matched normal tissues and cancer of the testis which showed increased 11β-HSD2 levels. Collectively, these results strongly suggest that dysregulation of the enzymes associated with local cortisol interconversion is a common feature of solid tumours.

Local cortisol levels regulate keratinocyte invasion, cohesion, and scattering in a two- and three-dimensional models of epidermal cancer

We hypothesized that the local production and degradation of cortisol could influence tumour progression. First, we demonstrated that cortisone, cortisol (hydrocortisone when used as a medication) and ACTH acted directly to increase the invasion of epidermal keratinocytes (**Figure 4A-D**). The effect was most striking amongst II-3 cells where there was a statistical increase in invasion following treatment with hydrocortisone and ACTH. The effect was not seen in HaCaT, I-7 or RT-3 cells.

Hydrocortisone has been shown to exert pro-adhesive effects in normal keratinocytes (Nguyen *et al*., 2004). As inhibition of 11β-HSD2 leads to increased cortisol levels in the culture medium, a dispase-based assay was undertaken to investigate whether inhibition of 11β-HSD2 altered intercellular adhesion in malignant epidermal cells. For this experiment we opted for a pharmacological block of the enzyme using 18β-GA because this inhibitor, unlike siRNAs, allowed to achieve consistent levels of 11β-HSD2 activity throughout the experimental period (not shown). As shown in **Figure 5A-C**, keratinocyte monolayers

displayed increased cell–cell adhesive strength (i.e., resistance to cell–cell detachment) in the presence of the 11β-HSD2 inhibitor 18β-GA. 11β-HSD2 activity was also examined in a three-dimensional model of cell adhesion. Multicellular aggregates were prepared in the presence or absence of 18β-GA (**Figure 5D, E**) and whilst the number of cells that aggregated together in suspension and formed MCAs after 24 hours of incubation was similar in both groups (not shown), intercellular cohesion of mature (24 hour) MCAs, as quantified by the release of single cells from the spheroids after mechanical stress, was higher in MCAs cultured in the presence of 18β-GA (**Figure 5H**). The MCAs were left to attach and disaggregate on a collagen substrate in 18β-GA-free medium and migration from the core of the cell aggregate was measured in a scattering assay. Control MCAs were almost entirely dissolved 6 hours after seeding (**Figure 5F)** whereas MCAs grown in the presence of 18β-GA displayed a reduced ability to scatter and migrate from the spheroids after attachment on collagen substrates (**Figure 5G**) and were able to cover only ~55% of the area compared to control MCAs (**Figure 5I**).

Taken together, the data demonstrate that pharmacological modulation of cortisol degradation via 11β-HSD2 inhibition influences cell adhesion and three-dimensional intercellular cohesion of malignant keratinocytes.

Expression of 11β-HSD2 in skin and its down-regulation in squamous cell carcinoma

The above results suggested that 11β-HSD2 expression was important to the bioavailability of tumour-derived cortisol. However, previous research on the expression of 11β-HSD2 in the human integument has given conflicting results (Tiganescu *et al*. 2011, Vukelic *et al*.

2011, Terao *et al*. 2013). In this study, we used a rabbit antibody against the residues 261-405 of human 11β-HSD2 at a 1:100 dilution, a concentration at which normal skin displayed strong staining (**Supplementary Figure 4**). Using tissue arrays, immunohistochemical expression of 11β-HSD2 was examined in 40 cases of squamous cell carcinoma (SCC) and 8 normal skin controls (**Figure 6**). The intensity of 11β-HSD2 staining in the malignant tissues was consistently less than control tissues. 11 of 40 SCCs (27.5%) displayed no staining whilst staining intensity was weak to moderate in a further 25 samples (62.5%). Overall, the 11β-HSD2 staining score in SCC was statistically less ($p<0.05$) than in the controls (**Supplementary Figure 5**). We therefore conclude that the expression of 11β-HSD2, a key enzyme in the local control of keratinocyte-derived cortisol, is significantly reduced in SCCs of the skin.

Taken together, the data demonstrate that cancer cells produce cortisol irrespective of their tissue of origin. Furthermore, 11β-HSD2-mediated regulation of tumour-derived cortisol potentially plays an important role in the pathobiology of epidermal malignancy.

Discussion

This study presents a series of observations that demonstrate an important role for the glucocorticoid system, and specifically for 11β-HSD2, in the physiology of normal and malignant epithelial cells. First, we show that cortisol production is a common feature of a broad spectrum of cancers and that cancer-derived cortisol inhibits CD8+ cytotoxic T lymphocyte proliferation in a GR-specific fashion. Second, we demonstrate that 11β-HSD1/2 expression is altered in many different cancer types in a fashion that is predicted to lead to a pro cortisol-producing phenotype. Third, we establish that normal human skin

tissues express detectable levels of 11β-HSD2 and that levels are reduced in SCCs. Fourth, we demonstrate *in vitro* that 11β-HSD2 is synthesized by human malignant keratinocytes under basal conditions and its expression and activity regulate both the production and inter-conversion of active cortisol. Finally, we show that 11β-HSD2 modulates intercellular adhesion and that inhibition of 11β-HSD2 by 18β-GA increases intercellular cohesion. .

A major finding of this study was that cancer cells produce cortisol regardless of their tissue of origin, strongly supporting the view that a cancer-associated glucocorticoid system is active in malignancy. Not only was cortisol production enhanced by ACTH treatment but also, analysis of CD8+ T cell responses revealed a linear correlation between basal cortisol levels in supernatants from cancer cells and inhibition of lymphocyte proliferation. Given the importance of T cell-mediated responses in the progression of cancer, as well as its prognostic relevance (Knutson & Disis, 2005; Ward *et al*., 2014), our data suggest that the production of cortisol by malignant cells may have an important immunoregulatory function. These findings are consistent with Sidler *et al*. (2011) who showed that tumour-derived GCs from colon cancer induced suppression of T-cell activation. The present study, however, is the first to extend this concept to a broad spectrum of solid tumours. It is also intriguing that under our experimental conditions, colorectal cancer cells (HT29, SW620, SWH80) were the only cell type not to show a correlation between cortisol production and inhibition of CD8 T lymphocytes. Specifically, these cells produced low amounts of basal cortisol but concurrently, reduced T cell proliferation. We have shown previously that other

tumour-derived molecules such as prostaglandins may be involved in the immune regulation of CD8 cells in cancer. (Ahmadi et al., 2008; Basingab et al., 2016).

In the past two decades, the intracellular inter-conversion of inactive and active steroids by the 11β-HSDs has emerged as a key mechanism underlying tissue-specific regulation of glucocorticoid action. In non-adrenal tissues, 11β-HSDs have been shown to play a crucial role in the control of basic physiological functions, as well as in disease processes such as osteoporosis (Cooper *et al*. 2008), insulin-resistance (Purnell *et al*. 2009), chronic periodontitis (Nakata *et al*. 2016) and cancer (Zhang *et al*., 2009; Rabbitt *et al*. 2003). With regard to squamous epithelia, a number of studies from Slominski's group have supported the hypothesis that the skin has the potential to act as a neuroendocrine organ (Slominski *et al*. 2006; 2007; 2011). We have extended these observations and demonstrated that squamous epithelium lining the oral cavity acts as a corticosteroid-producing system and that alteration of 11β-HSDs in oral keratinocytes may play an important role in disease (Cirillo *et al*. 2012). However, while studies addressing the function of 11β-HSDs in epidermal tissues support a key role for 11β-HSD type 1 in epidermal biology and pathophysiology (Hardy *et al*. 2006; Hennebert *et al*. 2007; Terao *et al*. 2011; Tiganescu *et al*. 2011, Lee *et al.* 2013, Itoi *et al.* 2013, Terao *et al.* 2014, Tiganescu *et al*. 2015), there is some disagreement about the putative expression and function of 11β-HSD type 2 (Cirillo and Prime, 2011; Vukelic *et al*. 2011; Tiganescu *et al*. 2011, Terao *et al*. 2013). In the reports by Tiganescu *et al*. (2009) and Terao *et al*. (2013), for example, the expression of 11β-HSD2 could not be demonstrated in human skin suggesting that keratinocytes cannot de-activate cortisol. In the present study, we demonstrated 11β-HSD2 in normal

skin specimens, findings that are consistent with our published data showing 11β-HSD2 at both the mRNA and protein levels in keratinocytes. We conclude that 11β-HSD2 is present in epidermal keratinocytes.

To the best of our knowledge, the data reported in the present study are the first to show that 11β-HSD2 is not only expressed by epidermal keratinocytes but, also, that the pharmacological manipulation of 11β-HSD2 modulates cortisol levels and regulates certain cellular functions. The question remains, however, as to whether 11β-HSD2 can be modified for therapeutic benefit. Interestingly, it has been shown recently that 18β-GA, administered either prophylactically or therapeutically, induces a dramatic reduction in bone loss using a mouse model of periodontitis (Sasaki *et al*., 2010). The authors suggested that this effect was independent of glucocorticoids because a down-regulation of 11β-HSD2 mRNA was not seen. 18β-GA, however, is known to impair 11β-HSD2 function by blocking enzymatic activity rather than by inducing down-regulation at the transcriptional level (Zhang et al. 2009; Kratschmar *et al*. 2011). In view of our recent findings (Cirillo et al., 2012), we tentatively suggest that 18β-GA may reduce tissue inflammation and limit the bone loss that occurs in periodontal disease by increasing local cortisol levels. Further studies, however, are required to verify this proposal..

Our data demonstrate that silencing of 11β-HSD1 correlates with a temporary decrease in the local bioavailability of active cortisol, which increases once 11β-HSD1 expression is restored. The reverse is true for 11β-HSD2 where expression of the enzyme inversely correlates with cortisol levels. Therefore, alteration of 11β-HSD1/2 expression and

function is tightly linked to the levels of cortisol in the microenvironment. In the present study, we examined the expression of 11β-HSD enzymes in a broad spectrum of cancer-types using an open IHC database. We show that dysregulation of 11β-HSDs is common in a broad spectrum of cancer-types; the enzymes were either up-regulated (11β-HSD1) or down-regulated (11β-HSD2), or both, in 11 out of 15 cancer types from 315 cases; the trend was consistent with an increase in the bioavailability of local cortisol. In SCC of the skin, there was a significant down-regulation of 11β-HSD2 compared to normal skin., findings that are in accord with results from colorectal (Žbánková *et al*., 2004) and oral (Cirillo *et al*., 2012) cancers, but contrast with osteosarcoma (Bland *et al*., 1999) and ovarian cancer (Temkin *et al*., 2006). The subject has recently been extensively reviewed (Azher et al., 2016).

Recently, it has been shown that corticosteroids suppress key components of the senescence-associated secretory phenotype (SASP) (Laberge *et al*., 2012). In epithelial cells, cellular senescence is a mechanism that suppresses the early stages of cancer development suggesting that tumour-derived cortisol may inhibit the anti-cancer activity of the SASP and favour cancer progression. By contrast, we have shown that the SASP of cancer-associated fibroblasts (CAFs) exerts pro-tumorigenic paracrine effects on malignant oral epithelial cells (Hassona *et al*., 2013; 2014). In these circumstances, cortisol produced by malignant epithelial cells would be expected to have paracrine tumour suppressive effects. Interestingly, our recent observations have shown that cortisol is produced by normal fibroblasts (Cirillo et al., 2012) as well as cancer associated fibroblasts (Cirillo N, unpublished observations). Studies are on-going to investigate the role of

cortisol in the tumour micro-environment and the inter-relationship between epithelial cells, fibroblasts and immune cells.

In conclusion, we report that a non-adrenal glucocorticoid system is active in cancer and that the resulting cortisol production suppresses lymphocyte proliferation. Further, we show that 11β-HSD2 modulates intercellular cohesion and that inhibition of 11β-HSD2 leads to an increase of secreted cortisol in epidermal cells. 11β-HSD2 is down-regulated in SCCs of the skin as well as in 8 other cancer types. We suggest that 11β-HSD2 could be used as a potential biomarker of tumour progression and may be of value therapeutically by regulating local tissue concentrations of cortisol in skin disease.

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Legend to figures

Figure 1. Naïve TcR transgenic tumour antigen-specific CL4 CD8+ T lymphocytes were incubated for 24 hours with supernatants of ACTH-treated II-3 cells in the presence the GR inhibitor RU486, or without treatment (A). CD8+ lymphocytes were incubated with supernatants from 12 cancer cells with known basal cortisol production. The cells were counted after 24 hours and their number was plotted against the corresponding cortisol levels in the supernatants (B,C).

Figure 2. Cortisol levels in the supernatant of un-stimulated cells (Bsl) and cells treated with 10nM ACTH for 24 hours (ACTH) were assessed by ELISA (A). Western blotting and immunofluorescence were undertaken to investigate the expression of 11β-HSD1 and 11β-HSD2 proteins (B). siRNA was used to transiently knock-down the expression of 11β-HSD1 (C) and 11β-HSD2 (D) in II-3 cells; in parallel, the protein level of these enzymes was assessed by Western blotting. Silencing of 11β-HSD1 resulted in a reduced ability of II-3 to activate cortisone (100nM) into cortisol (C). Silencing of 11β-HSD2 in HC-treated cells (100nM) resulted in an increase in the concentration of active cortisol in supernatants (D). Both effects were transient and progressively reverted when the expression levels of 11β-HSDs were restored. Both enzymes were detectable by IHC in human skin cancers, namely SCC (E) and melanoma (F).

Figure 3. Staining intensity of 11β-HSD1/2 using a publicly available dataset featuring 15 different normal/malignant tissues was computed and illustrated by histogram. *p* value of paired *t* test is reported. Blue colour indicates up-regulation whereas red colour indicates down-regulation.

Figure 4. HaCaT (A), I-7 (B), II-3 (C), and RT-3 (D) were used for the invasion assay in the presence of 100nM cortisone, 100nM hydrocortisone, 10nM ACTH or without treatment (Control). ANOVA statistical analysis was used to determine the significance of the results. The histograms show mean values of four independent experiments ± standard deviation.

Figure 5. A dispase-based assay was undertaken to assess cell-cell adhesion strength in II-3 cells with or without treatment with 18β-Glycyrrhetinic Acid (A-C). Cohesion strength (D, E, H) and scattering (F, G, I) of 3-D cellular aggregates in the presence or absence of 18β-GA was also assessed.

Figure 6. Staining of 11β-HSD2 at a 1:100 dilution was undertaken using tissue microarrays. The array is shown with (a) and without (b) IHC staining and shows cases of squamous cell carcinoma (n=40), normal skin tissues (n=8) and a positive control (melanoma). Typical aspects of normal (c, e) and cancerous (d, f) samples are reported at 4x and 20x magnification.