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Colon cancer-derived myofibroblasts increase endothelial cell migration by glucocorticoid-sensitive secretion of a pro-migratory factor

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ABSTRACT

Angiogenesis is important in cancer progression and can be influenced by tumor-associated myofibroblasts. We addressed the hypothesis that glucocorticoids indirectly affect angiogenesis by altering the release of pro-angiogenic factors from colon cancer-derived myofibroblasts.
Our study shows that glucocorticoids reduced prostanoids, urokinase-type plasminogen activator (uPA) and angiopoietin-like protein-2 (ANGPTL2) levels, but increased angiogenin (ANG) in supernatant from human CT5.3hTERT colon cancer-derived myofibroblasts. Conditioned medium from solvent- (CMS) and dexamethasone (Dex)-treated (CMD) myofibroblasts increased human umbilical vein endothelial cell (HUVEC) proliferation, but did not affect expression of pro-angiogenic factors or tube-like structure formation (by HUVECs or human aortic ECs). In a HUVEC scratch assay CMS-induced acceleration of wound healing was blunted by CMD treatment. Moreover, CMS-induced neovessel growth in mouse aortic rings *ex vivo* was also blunted using CMD. The latter effect could be ascribed to both Dex-driven reduction of secreted factors and potential residual Dex present in CMD (indicated using a dexamethasone-spiked CMS control). A similar control in the scratch assay, however, revealed that altered levels of factors in the CMD, and not potential residual Dex, were responsible for decreased wound closure.
In conclusion, our results suggest that glucocorticoids indirectly alter endothelial cell function during tumor development *in vivo*.
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1. Introduction

Angiogenesis, the formation of new blood vessels from an existing vascular network [1], is essential for embryonic growth. In healthy adults angiogenesis is restricted to discrete physiological processes (e.g. the regulation of the reproductive tract, muscle growth) and contributes to wound healing [2]. Excessive or impaired angiogenesis has also been implicated in disease pathogenesis (e.g. in malignant or inflammatory disorders [2]), and is associated with promotion of tumor growth and metastasis. Consequently, the potential of angiogenesis as a therapeutic target (e.g. in cancer [1–3], retinopathy [4] and tissue ischemia [5]) has attracted considerable research interest.

Tumors use blood vessels not only as a source of nutrients and oxygen, but also to transport cancer cells to establish a new, metastatic site [6]. Cancer cells can directly modulate angiogenesis via secretion of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), angiopoietins, basic fibroblast growth factor (bFGF), interleukins (ILs) or transforming growth factors (TGFs) [3,7]. Epithelial tumors consist of cancer cells and a surrounding microenvironment composed of an extracellular matrix, stromal cells, inflammatory cells and endothelial cells (ECs). All these components play an important role during tumor development [8]. Cancer-associated fibroblasts (myofibroblasts) are present at the invasive edge of the tumor and share properties of both smooth-muscle cells and fibroblasts. Myofibroblasts, which are essential during wound healing and embryonic development [9], can also influence tumor progression [10,11] either directly, through paracrine signaling to cancer cells, or indirectly, by modulation of protease activity, modulation of extracellular matrix remodeling, and recruitment of

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immune cells [8,10]. Myofibroblasts also have the potential to alter EC function and influence tumor angiogenesis [8,11]. In breast cancer, cancer-associated fibroblasts promote vascularization by recruiting endothelial progenitor cells to the tumor via increased release of stromal-cell derived factor 1 (SDF-1) [12]. Moreover, prostaglandin (PG)E_2-stimulated intestinal sub-epithelial myofibroblasts display an increased expression of vascular endothelial growth factor (VEGF) and hepatocyte growth factor/scatter factor (HGF/SF), which promote EC migration [13].

Glucocorticoids (GCs) are steroidal ligands of the glucocorticoid receptor (GR), which belongs to the nuclear receptor superfamily. Stimulation of GR regulates many physiological processes, mainly via gene transactivation or transrepression [14]. Consequently, glucocorticoids are clinically important as potent anti-inflammatory compounds in treatment of autoimmune diseases [15], and as adjuvants in cancer therapy [16]. Moreover, GCs provide an effective treatment of infantile hemangiomas (IHs) [17]. GC-mediated inhibition of angiogenesis is well-documented [18] and has therapeutic potential in the treatment of cancer [19,20]. The direct, growth-inhibitory influence of GCs on vascular smooth muscle cells is well-established [21,22]. Furthermore, more recent investigations have demonstrated GR-dependent, GC-mediated inhibition of tube-like structure formation by ECs *in vitro*, independent of GCs' anti-inflammatory actions [23]. GCs can also inhibit angiogenesis indirectly by suppression of pro-angiogenic factors, such as VEGF and IL-8, produced by prostate cancer cells [20], and possibly by extracellular matrix degradation or modification of cytokine production [24].

We recently reported that GCs regulate myofibroblasts, decreasing production and secretion of a number of factors linked to cancer progression and invasion: tenascin C (TNC), TGFβ, HGF/SF [25–27]. These factors are all known to also affect the angiogenic response through a number of mechanisms [28–30]. Combined with our data, these studies suggest that GCs could have the ability to inhibit myofibroblast-induced stimulation of angiogenesis by altering the composition of the myofibroblast secretome. Therefore, this investigation addressed the hypothesis that exposure of colon cancer-derived myofibroblasts to GCs can reduce secretion of angiogenic factors and thus inhibit their ability to promote pro-angiogenic changes in ECs.

2. Materials and methods

2.1. Cells and reagents

Human stromal colon cancer-derived myofibroblasts (CT5.3hTERT cells) were isolated as described [26,31] and cultured (37 °C, 10% CO_2) in Dulbecco's modified Eagles Medium (DMEM; Life Technologies, Merelbeke, Belgium) supplemented with 10% fetal calf serum (Greiner bio-one, Wemmel, Belgium), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Life Technologies). Primary human umbilical vein endothelial cells (HUVEC; Promocell, Heidelberg, Germany) and human aortic endothelial cells (HAoEC; Promocell) were cultured in Endothelial Cell Growth Medium-2 (EGM2; Lonza, Wokingham, UK), containing all manufacturer-supplied supplements (2% FCS, 0.1% VEGF, 0.4% hFGF-2, 0.1% R^3-IGF-1, 0.1% hEGF, 0.1% ascorbic acid, 0.1% heparin, 0.1% GA-100) except hydrocortisone. HUVECs were cultured (37 °C, 5% CO_2) on 0.1% gelatin-coated flasks and were studied between passages 2 and 7. In experiments we used EGM2 containing 2% FCS or 0% FCS, abbreviated respectively $EGM2^{S+}$ and $EGM2^{S-}$.

Dexamethasone (Dex), hydrocortisone (Hcrt), prednisolone (Pred), fluocinolone acetonide (FA) and the GR antagonist RU38486 (RU) were purchased from Sigma-Aldrich (Diegem, Belgium). All reagents were dissolved in ethanol and used at a final concentration of 1 μM, except RU (2 μM). A selective GR modulator (SEGRM), compound A (CpdA) was prepared as previously described [32] and used at a final concentration of 10 μM. The total solvent concentration (maximally 0.1%) was consistent in all conditions.

2.2. Conditioned medium preparation

Conditioned medium (CM) was obtained from 10×10^6 CT5.3hTERT myofibroblasts and prepared as described [26]. Briefly, cells were washed three times with serum-free DMEM and treated for 48 h with solvent (ethanol), Dex (1 μM), Hcrt (1 μM), Pred (1 μM), CpdA (10 μM) or RU (2 μM) in serum-free DMEM. After this incubation CM was collected, concentrated 10-fold using centrifugal filter tubes with a 3 kDa cut-off (Amicon Ultra, Merck Millipore, Darmstadt, Germany), filter-sterilized (0.2 μm pore size) and stored (−20 °C) for subsequent functional and biochemical assays. For functional assays CM from solvent and Dex-treated myofibroblasts (CMS and CMD, respectively) were diluted with $EGM2^{S+}$ or $EGM2^{S-}$ or with serum-free DMEM prior to treatment. Taking into account the concentrating procedure of CM and further dilution in the functional assays, the maximal final concentration of Dex in the CMD treatment was calculated to be 50 nM. CM concentrations and dilutions used in particular experiments are listed in Supplementary Table 1.

2.3. Protein analysis: protein array, Western blot and immunoassay (ELISA)

CM from CT5.3hTERT myofibroblasts treated with Dex or solvent (CMS and CMD, respectively) were collected after 48 h, 4-fold concentrated and subjected to Ray Bio® Biotin Label-based Human Antibody Array I (Raybiotech, GA, USA, cat no: AAH-BLM-I-2) which allows simultaneous analysis of expression levels of 507 human target proteins (including cytokines, chemokines, adipokines, growth factors, angiogenic factors, proteases, soluble receptors and soluble adhesion molecules) in cell culture supernatants. The assay was performed according to the manufacturer's instructions with the results visualized using X-Ray films (GE Healthcare, Diegem, Belgium) and the signal evaluated using ImageJ software [33]. For further analysis, we set the threshold value for the ratio between relative protein signals in CMS vs. CMD as > 1.5. Selected factors analyzed using the protein array are listed in Supplementary Table 2.

For further validation of the protein array results, CT5.3hTERT myofibroblasts were incubated for 48 h with steroids (Dex, Hcrt, Pred; 1 μM), CpdA (10 μM), RU (2 μM) or solvent. Conditioned media were collected, concentrated (10-fold) and protein concentrations were evaluated using the Lowry method [34]. Samples were prepared in SDS sample buffer (50 mM Tris pH 6.8; 2% SDS; 10% glycerol; bromophenol blue; 100 mM DTT), loaded (25 μg) onto an SDS-PAGE gel and subjected to the standard Western blot protocol, as described by Santa Cruz (Santa Cruz, Heidelberg, Germany). The proteins were probed using the following primary anti-human antibodies: anti-uPA (H-140) (1/500, Santa Cruz Biotechnology, cat no: sc-14019), anti-ANG I (H-123) (1/500, Santa Cruz Biotechnology, cat no: sc-9044) and anti-ANGPTL2 (P-13) (1/500, Santa Cruz Biotechnology, cat no: sc-107143). Results were visualized using species-specific HRP-linked secondary antibodies and reagents: anti-rabbit (1/4000, GE Healthcare, cat no: NA934V), anti-goat (1/3000, Santa Cruz Biotechnology, cat no: sc-2020), ECL solution (Thermo Scientific, Gent, Belgium) and X-Ray films (GE Healthcare). Signal quantifications were performed using ImageJ software [33].

The internalization and subsequent degradation of the acetylated low density lipoprotein (Ac-LDL) is a characteristic feature of endothelial cells. In order to evaluate whether the conditioned medium from myofibroblasts affects the basic endothelial character of HUVECs, we performed an Ac-LDL uptake assay. Briefly HUVECs were incubated for 24 h in $EGM2^{S+}$ (control), DMEM, CMS or CMD. DMEM and 10-fold concentrated CM were diluted 1:1 with $EGM2^{S+}$. An Ac-LDL assay was then performed, as described (see Supplementary methods in Supporting Information).

In order to determine the concentrations of prostanoids in conditioned medium from myofibroblasts and HUVECs, and in HUVEC lysates, we performed immunoassays (ELISAs) for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostacyclin (PGI_2; by assessing 6-keto-$PGF_{1\alpha}$) and

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