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Tumor Necrosis Factor Alpha Production is Increased *In Vitro* Bearing the R151C Variant of *MC1R* Gene

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Abstract

Background: *MC1R* (Melanocortin 1 Receptor) is considered a moderate penetrance susceptibility gene for Malignant Melanoma (MM). Bearing a MC1R variant carries a 2.2 to 4.8-fold risk of developing it. *MC1R* is a major determinant gene for skin pigmentation and its ligand, the melanocyte stimulating hormone (αMSH), also presents anti-inflammatory properties. Agonism of αMSH on a wildtype MC1R, but not on mutated MC1R, decreases invasion, tumour necrosis factor (TNFα)-stimulated invasion and pro-inflammatory cytokine-stimulated activation of the NF-kB transcription factor in MM cell lines.

Aim: The aim of the present work was to investigate the secretion of TNFα secondary to the interaction of the αMSH on *MC1R* variants commonly associated with MM susceptibility.

Methods and Results: We determined TNF-α secretion in human kidney cells (HEK293T) transiently transfected with a vector containing the consensus sequence and the variants of *MC1R* gene: D84E, R151C, R160W, R163Q. By immunofluorescence microscopy and real time PCR we determined expression of MC1R in all cells transfected. We observed that those cells that contained a mutated MC1R (R151C) presented an increased secretion of TNF-α to the media, measured by an immunosorbent assay ELISA, with respect to the WT ($p=0.003$). This effect was not seen in the cells that contained the other mutated constructs ($p>0.05$).

Conclusions: Our results suggest that the αMSH agonism on a mutated MC1R with the variant R151C in HEK293T cells might be associated with increased TNF-α production.

Keywords: Melanoma; MC1R; TNF-α; Variants

Introduction

Malignant Melanoma (MM) is responsible for 1-2% of all deaths from cancer [1]. It is the most common cause of death among the skin diseases and mortality is determined by the development of metastases. *MC1R* (Melanocortin 1 Receptor) gene, which encode for melanocyte stimulating hormone (αMSH) receptor, is currently considered a moderate penetrance susceptibility gene for MM [2]. MC1R is a major determinant of skin pigmentation [3] but growing evidence indicates that MC1R and its ligand αMSH have several other functions: anti-inflammatory properties (including inhibition of tumour necrosis factor alpha, TNFα [4]), modulation of oxidative stress or inhibition of extracellular matrix. Bearing a MC1R variant carries a 2.2 to 3.9-fold risk of developing melanoma; carriers of two variants show a 4.1 to 4.8 fold [2,3].

Specifically, R151C variant has been found repeatedly to be associated with MM development with OR mostly ranging from 1.69 (95% CI= 1.12-2.55) to 2.76(95% CI= 1.59-4.78) [2,4,5].

Agonism of αMSH on a wildtype MC1R decreases invasion, TNFα-stimulated invasion and pro-inflammatory cytokine-stimulated activation of the NF-kB transcription factor in MM cell lines [6,7]. However, in MM cell lines the agonism of the αMSH on a MC1R bearing the R151C variant does not reduce cell migration, neither pro-inflammatory cytokine-stimulated activation of the NF-kB transcription factor [7]. Agonism on a wildtype MC1R inhibits the secretion of TNFα [8] but interestingly in MM cell lines, the TNFα increases the capacity of adhesion of the cells [9], promotes their migration [10] and their invasiveness [6]. Thus, the aim of the present work is to investigate TNFα secretion secondary to the interaction of the αMSH on MC1R variants commonly associated with MM susceptibility.

Methods

Cell Lines

HEK293T cells were grown in 24-well plates using Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), 2mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin, with 5% CO_2 at 37ºC. Cells were grown to 80% confluence and then transfected. All experiments were performed in independent duplicate or triplicate assays.

DNA Constructs and Transfections

The cDNA for consensus or variant human *MC1R* alleles (D84E, R151C, R160W, R163Q) were generated as HindIII-EcoRI tagged, Pfu (iproof HF, BioRad) amplified PCR fragments and cloned into the pcDNA3.1 expression vector (Invitrogen, CA, USA). The variant human MC1R D84E, R151C, R160W and R163Q were chosen due to its relevance in the literature and previous findings [3-5]. Non-transfected HEK293T cells were used as a negative control. Cells were transfected using DNA transfection reagent JetPrime™ (Polyplus transfection, France) and assayed 24-48 h after transfection.

Immunofluorescence

Cells were cultured on Poly-L-lysine treated coverslips. Forty-eight hours after transfection unpermeabilized cells were fixed in 4% paraformaldehyde and stained with MC1R Ab (N-19) (Santa Cruz Biotechnology) 1:100 in blocking solution (BSA 5% in PBS) overnight at 4ºC. Then, bound MC1R was visualized with a 1:400 FITCT-bovine anti-goat secondary antibody (Jackson Immuno Research Laboratories). In a second assay cells were permeabilized 5 min with PBS-T (0.1% Triton X-100). Coverslips were mounted using fluoroshield medium with DAPI (Sigma-Aldrich). Cells images were obtained with a Leica DM6000B epifluorescence microscope. Quantification of cell surface fluorescence was performed by selection of representative unpermeabilized cells expressing each variant receptor. Representative 6-10 pictures per coverslip were taken of unpermeabilized cells (at least four cells per independent experiment).

TNF-α Measurement

Cultured cells were stimulated for the TNF-α production. Forty-eight hours later, the medium was replaced with serum-free DMEM and NDP-αMSH was added at different concentrations (10^{-5}, 10^{-10} and 10^{-15} M) and incubated for 1 hour. Then 30 ng/ml of PMA (phorbol 12-myristate 13-acetate) and different concentrations of LPS (10,100 and 500 ng/ml) were added and incubated for additional 6 hours. Cells were recovered for the posterior measure of secreted TNF-α. Human TNF-α was measured by non-competitive Enzyme-Linked Immunosorbent Assay (ELISA), using a commercial kit (R&D Systems Inc.) following the manufacturer's instructions. The within-assay coefficient of variation for all assays was less than 10%.

MC1R Expression by Real -Time PCR

RNA Extraction

Cells RNA was extracted with the High Pure RNA extraction Kit (Va en mayúsculas?). cDNA was synthesized using M-MLV Reverse Transcriptase (Promega). Real-time quantitative PCR amplifications (qPCR)reaction mix contained: 1X IQTM SYBR Green Supermix (BioRad, Hercules, CA, USA), 0.25 µM of each primer and 40 ng/µl of template cDNA in a final volume of 20 µl. Amplification was performed on an iCycler iQ real-time detection PCR system (BioRad, Hercules, CA, USA): Step 1: 5 min at 95ºC, Step 2: 40 cycles (95ºC for 20 sec, 60ºC for 20 sec and 72ºC for 20 sec). To confirm amplification specificity, PCR products were subjected to a melting curve analysis (Step 3: 55-95ºC with a gradient of 0.5ºC). ACTB was used as reference gene and MC1R oligonucleotides were: FW (5`-ACATCTCCATCTTCTACGCAC-3`) and RV (5`-TGGTCGTAGTAGGCGATGAA-3`) (Invitrogen). The quantification data was analysed with the iCycler analysis software. All the reactions were performed on triplicates. The relative mRNA amounts were calculated using the "ΔΔCp with efficiency correction" calculation method [11].

Results

Immunofluorescence Detection of Cell Surface Expression of MC1R

The MC1R wildtype and the variant alleles (D84E, R151C, R160W and R163Q) constructs were transiently transfected to human embryonic cells HEK293T and the surface expression on unpermeabilized cells was determined by the detection of a MC1R antibody (N-19). As a control, we used permeabilized cells to confirm the specificity of the antibody. Quantification of cell surface fluorescence of MC1R variants with respect to the wildtype

indicated no significant differences between them, except for the variant R151C ($p<0.001$) which was less intense (Figure 1).

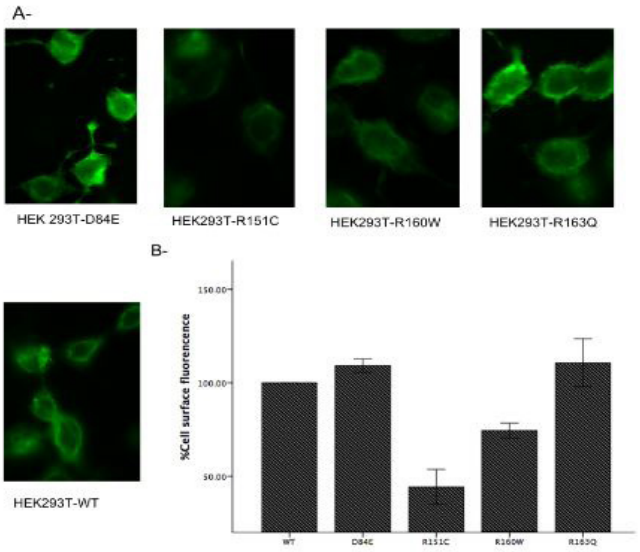

Figure 1: Cell surface MC1R antibody binding in transient cell HEK293T expression of MC1R WT and variant alleles D84E, R151C, R160W and R163Q (columns left to right). (A) Immunofluorescence images of unpermeabilized cells stained for MC1R. Cells transfected with MC1R-WT-FITCT, MC1R-D84E-FITCT and MC1R-R163Q-FITCT show similar cell surface staining but cells transfected with MC1R-R151C-FITCT showed a less intensely signal. All images are representative of at least three independent experiments. (B) Quantification of MC1R cell surface fluorescence intensities were performed using ImageJ software. Comparisons of variant MC1R image intensity are expressed relative to WT, which is set at 100%. The error bars are representative of +SD for two independent experiments. T-Student test was used for comparisons: $p<0.001$ between cells with the WT and cells with the variant R151C; no significant differences were observed between WT and the other variants ($p>0.05$). Cells with the variant R163Q presented the highest surface fluorescence intensities.

mRNA Expression Levels of *MC1R* Variants

MC1R mRNA expression was assessed in HEK293T cells transfected with the wildtype and variants of MC1R constructs using real-time qPCR. All cells expressed MC1R without significant differences between them ($p>0.05$) (data not shown).

TNF-alpha Secretion in Transfected Cells

We observed that treatment with 10^{-15} M NDP-αMSH and LPS (100ng/ml) in cells transfected with the R151C variant increased the secretion of TNF-α when compared to WT ($p=0.003$). Cells transfected with MC1R constructs with the other variants or the wildtype did not show a significant increment of the cytokine levels (Figure 2).

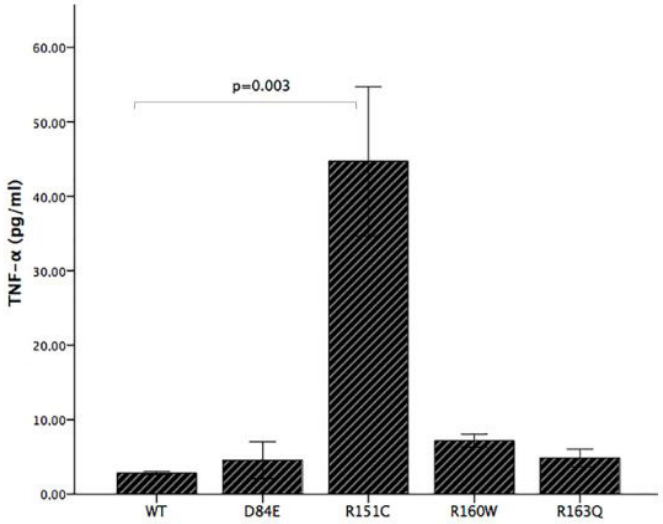

Figure 2: TNF-α release was measure in the cell medium by immunoassay ELISA. Cells transfected with the R151C variant (treated with 10^{-15} M NDP-αMSH and 100ng/ml of LPS) showed increased secretion of TNF-α. Equivalent supernatant levels of TNF-α can be seen for the other cell variants constructs ($p>0.05$). The error bars displayed are the standard deviation of duplicate readings performed from at least two independent experiments.

Discussion

The significant association between MC1R variants and MM susceptibility is well known [2,4]. It is often discussed in terms of decreased eumelanogenesis and the correspondent reduction in protection against the known deleterious effects of UV radiation. But multiple data suggest the modulation of the UV-induced DNA damage by MC1R [12,13] and that effect of αMSH on DNA repair enables melanocytes to survive not strictly determined by increased melanin content [14]. These mechanisms need expression of functional MC1R [15].

In MM cell lines, TNF-α increases the capacity of adhesion of the cells [9], promotes their migration [10] and their invasiveness [6]. Expression of integrins by MM cells is observed along initial spread of the disease [9] but αMSH through its receptor MC1R decreases the expression of TNFα-mediated ICAM-1 in melanocytes, melanoma cells, and keratinocytes [16]. Anti-inflammatory effects of activated MC1R seem to need also a functional receptor as the agonism on a mutated MC1R does not reduce TNF-α cytokine-stimulated activation of the NF-kB transcription factor in MM cell lines [7]. Current results showing increased *in vitro* levels of TNF-α level in HEK293T cells secondary to agonism of αMSH on MC1R bearing R151C variant deserve further studies not only on melanocytes but on keratinocytes as ability of melanocytes to transform into melanoma cells seems

to be partially regulated by the later [9]. When MM develops, the keratinocytes adjacent to primary tumour stain intensively with anti-MC1R antibodies [17] with a gradient increasing towards the tumour [18]. Thus, R151C carriers would boost TNF-α secretion around growing MM, favouring inflammation and invasiveness. Garcin, et al. published TNF-α expression was not inhibited after stimulating HaCaT cells transfected with the R151C variant [19]. Although slightly increased, there were no significant differences compare to expression by HaCaT cells with wild MC1R receptor. On the other hand, HaCaT cells with wild MC1R construct and high MC1R expression decrease TNF-α levels efficiently. Although a disagreement may be initially considered between Garcin's and current results, both studies show an impaired TNF-α expression after agonism on R151C MC1R: No inhibition in HaCaT cells and increased levels in HEK293T cells.

D84E, R160W, R163Q variants didn´t increase TNF-α levels production after αMSH - MC1R agonism. This is not surprising; as R and r mutations impair MC1R function through different pathways (R163Q variant strongly impairs ERK activation although dose-response curves for agonist induced cAMP and the maximal stimulation appear normal [20]). Even on the cAMP pathway published data suggest different mechanisms (decreased cell surface expression seems the primary cause of loss of function for D84E, R160W and R151C; however, the RHC alleles R142H and D294H are ordinarily expressed and bind agonists with high affinity [13]). In HEK293T cells, agonism of αMSH on MC1R exerts a heterogeneous action on TNF-α *in vitro* secretion depending on mutational status and R151C variant seems to be associated with increased production. Further studies should assess results in melanocyte and keratinocyte cell lines.

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Conflicts of Interest

There are no conflicts of interest.

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