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Modulation of human monocyte/macrophage activity by tocilizumab, abatacept and etanercept: An *in vitro* study

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ABSTRACT

Tocilizumab, etanercept and abatacept are biological drugs used in the therapy of Rheumatoid Arthritis (RA). Their mechanism of action is well documented but their direct effects on human monocytes/macrophages have not been fully investigated. The objective of this study was to evaluate *in vitro* the influence of these drugs on monocytes/macrophages from healthy volunteers.

Human monocytes were isolated from healthy anonymous volunteers and cultured as such or differentiated to monocyte-derived macrophages (MDMs). The effect of tocilizumab, etanercept and abatacept (at concentrations similar to those in plasma of patients) on superoxide anion production, matrix metalloproteinase-9 (MMP-9) gene expression and activity, Peroxisome Proliferator-Activated Receptor (PPAR)γ expression and cell phenotype was evaluated.

Exposure of monocytes/macrophages to tocilizumab, etanercept or abatacept resulted in a significant decrease of the PMA-induced superoxide anion production. Interestingly, the expression of PPARγ was significantly increased only by tocilizumab, while etanercept was the only one able to significantly reduce MMP-9 gene expression and inhibit the LPS-induced MMP-9 activity in monocytes. When etanercept and abatacept were added to the differentiating medium, both significantly reduced the amount of $CD206^+$ MDM.

This study demonstrates that etanercept, abatacept and tocilizumab affect differently human monocytes/macrophages. In particular, the IL-6 antagonist tocilizumab seems to be more effective in inducing an anti-inflammatory phenotype of monocytes/macrophages compared to etanercept and abatacept, also in light of the up-regulation of PPARγ whose anti-inflammatory effects are well recognised.

1. Introduction

In the last fifteen years a number of biological disease-modifying anti-rheumatic drugs (bDMARDs) with proven efficacy have been developed. These drugs are structurally unrelated and have different pharmaco-dynamic and pharmacokinetic properties, although their clinical development has been largely overlapping and their differences have not been thoroughly investigated.

We have previously demonstrated that dexamethasone, indomethacin, and rofecoxib differ largely in their ability to inhibit NF-kappaB activity and/or TNF-alpha release from human monocytes (Lavagno et al., 2004), two mechanisms related to their efficacy. Furthermore, we have also shown that monocytes/macrophages from RA patients under synthetic disease-modifying anti-rheumatic drugs (sDMARD) treatment with less active disease present higher PPARγ protein expression and lower MMP-9 activity than RA patients with more severe disease. In analogy, when probing monocytes from healthy volunteers *in vitro*, methotrexate and methylprednisolone increase *in vitro* PPARγ protein expression and inhibit LPS-induced MMP-9 activity (Palma et al., 2012).

Etanercept, abatacept and tocilizumab are bDMARDs with proven efficacy in RA (Weinblatt et al., 2008; Kremer et al., 2005; Nishimoto et al., 2004) albeit with different mechanism of actions. Etanercept is a dimeric fusion protein that binds and inactivates soluble and cell-bound TNFα and lymphotoxin α (Mohler et al., 1993). Abatacept is a soluble recombinant fusion protein comprising the extracellular domain of human CTLA-4 that employs the high binding avidity of CTLA-4 for CD80/CD86 on APCs, to prevent full T cell activation (Schweitzer and Sharpe, 1998).

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Tocilizumab is a humanized anti-IL-6R antibody that prevents IL-6 from binding to its receptors, thereby blocking the pro-inflammatory effects of IL-6 (Md Yusof and Emery, 2013).

We have recently observed that RA patients in treatment with tocilizumab (n=7) have higher PPARγ monocyte expression compared to healthy donors (JAO, personal communication), paralleling the observations with sDMARDs. Capitulating on this exploratory finding, we have now examined whether etanercept, tocilizumab and abatacept differ in their ability to modulate human monocytes (isolated from healthy volunteers) and monocytes-derived macrophages responsiveness and phenotype.

2. Materials and methods

2.1. Cell culture

Human monocytes were isolated from 10 healthy anonymous human buffy coat samples (provided by the Transfusion Service of Busto Arsizio, Varese, Italy). For cell isolation, standard techniques of dextran sedimentation and Histopaque (density=1.077 g cm^{-3}) gradient centrifugation (400xg, 30 min, room temperature) were used. Cells were then recovered by thin suction at the interface, as described previously (Lavagno et al., 2004). Isolated cells were then re-suspended in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS), 2 mM glutamine and antibiotics; purified monocyte populations were obtained by adhesion (90 min, 37 °C, 5% CO_2). Cell viability (trypan blue dye exclusion) was usually >98%. Freshly isolated monocytes were also cultured in 10% FCS-enriched medium (RPMI 1640 medium containing 10% FBS, glutamine and antibiotics) for seven days and differentiated to monocyte-derived macrophage (MDM), defined as M0.

2.2. Cell viability

To assess potential drug toxicity in monocytes and MDM, cell viability was evaluated using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. Cells (1×10^5 cells) were challenged for 12 h and 7 days with concentrations compatible with those found in plasma (PC) of arthritic patients: abatacept 30 μg/ml (Kremer et al., 2005), etanercept 2 μg/ml (Keystone et al., 2004), tocilizumab 1 μg/ml (Nishimoto et al., 2008); then, the medium was replaced by the MTT assay solution (1 mg/ml; 2 h, 37 °C 5% CO_2). Supernatant was removed and DMSO was added in order to dissolve the purple formazan; the absorbance was read at 580 and 675 nm.

2.3. Superoxide anion (O_2^-) production

Monocytes (1×10^6 cells/plate) were treated for 1 h with the drugs in study and then stimulated with phorbol 12-myristate 13-acetate (PMA) 10^{-6} M for 40 min. O_2^- production was evaluated by the superoxide dismutase-sensitive cytochrome C reduction assay and expressed as nmol cytochrome C reduced/10^6cells/40 min, using an extinction coefficient of 21.1 mm, and as % inhibition of PMA-superoxide anion production. To avoid interference with spectrophotometrical recordings, cells were incubated with RPMI 1640 without phenol red, antibiotics and FBS.

2.4. Matrix Metalloproteinase (MMP)-9 gene expression and activity

Total mRNA was extracted from monocytes and MDM cells using GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich, MO, USA) according to manufacturer's instructions. First strand of cDNA was synthesised from 1 μg of total RNA using ImProm-II RT system (Promega). Real-time PCR was performed using GoTaq qPCR Master Mix (Promega) on an SFX96 Real-Time System (Biorad, Segrate, Italy). S18 ribosomal protein was used to normalize PCR product levels. Following oligonucleotide primers were used (from 5′ to 3′): S18 forward (Forw)-TGCGAGTACTCAACACCAACA, reverse (Rev) CTGCTT- TCCTCAACACCACA; MMP-9 Forw TCTTCCCTGGAGACCTGAGA Rev TTTCGACTCTCCACGCATCT.

MMP-9 activity was evaluated by Novex Gelatin Zymography (Invitrogen), according to the manufacturer's instructions. Monocytes were pre-treated with drugs for 6 h and then stimulated with LPS 500 ng/ml for 1 h. PG was used at 10 μM for 6 h; PPARγ antagonist GW9662 was used at 10 μM for 1 h. Supernatants were recovered and 10 μl were mixed with 10 ml Novex Tris-glycine SDS Sample Buffer (2X). Samples were run on a 10% Novex Zymogram Gelatin Gels (Invitrogen); protein bands were analysed with an image densitometer (Versadoc, Bio-Rad, USA).

2.5. PPARγ protein expression

PPARγ protein expression was evaluated in human monocytes and MDM by Western blot, as previously described (Palma et al., 2012). Cells were evaluated for their constitutive expression and after 6 h and 7 days challenge with the drugs at study. Cells were scraped off and lysed in radio-immunoprecipitation assay (RIPA) buffer. Protein samples (30 μg) were analysed by SDS-PAGE (10% acrylamide) and electro-blotted on nitrocellulose membranes. Immunoblots were performed using a cocktail comprising polyclonal rabbit anti-human PPARγ (Abcam, UK) and monoclonal mouse anti-human β-actin (Abcam, UK) antibodies. Chemiluminescence signals were analysed under non-saturating conditions with an image densitometer (Versadoc, Bio-Rad, USA). Semi-quantitative evaluation of PPARγ protein was performed by calculating the ratio between its expression and the expression of the reference housekeeping protein, β-actin. Results were expressed as fold increase relative to control, un-stimulated cells.

2.6. Flow Cytometric analysis

Measurement of phenotypic surface markers expression was performed by multi-parametric analysis by flow cytometry (FACS Calibur, BD) and analysed by CellQuest Software. Monocytes were treated for 12 h (to evaluate monocyte phenotype) or 7 days (to evaluate MDM phenotype) with abatacept, etanercept or tocilizumab. The following antibody panels were used: anti-CD16 (FITC, eBioscience), anti-CD36 (FITC, eBioscience), anti-CD86 (PE, eBioscience), anti-CD163 (PE, eBioscience), anti-CD206 (PerCp, eBioscience), and anti-CD14 (APC, eBioscience). The monocytes and MDM population were defined as CD14 positive cells. Data were therefore expressed as the number of $CD16^+$, $CD86^+$, $CD36^+$, $CD163^+$or $CD206^+$cells over the number of $CD14^+$cells. $Cd16^+$and $CD86^+$are M1-like marker, while CD36, Cd163 and Cd206 are M2-like marker. Comparison between treated and no-treated cells was performed and data were expressed as percentage of positive events.

2.7. Statistical analysis

Data were analysed by one-way ANOVA non parametric (Kruskal-Wallis). To adjust for multiple testing and to assess differences between control group and each of the treatment we have applied the Dunn's test. Data are expressed as means ± SEM of at least three independent experiments performed in triplicate. A value of $P < 0.05$ was considered significant.