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Research paper

Cell membrane-bound CD200 signals both via an extracellular domain and following nuclear translocation of a cytoplasmic fragment

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ABSTRACT

In previous studies we had reported that the immunosuppressive cell membrane bound molecule CD200 is released from the cell following cleavage by matrix metalloproteases, with the released soluble CD200 acting as an immunosuppressant following binding to, and signaling through, its cognate receptor CD200R expressed on target cells. We now show that although the intracellular cytoplasmic tail ($CD200_{C\text{-}tail}$) of CD200 has no consensus sites for adapter molecules which might signal the $CD200^+$ cell directly, cleavage of the $CD200_{C\text{-}tail}$ from the membrane region of CD200 by a consensus γ-secretase, leads to nuclear translocation and DNA binding (identified by chromatin immunoprecipitation followed by sequencing, Chip-sequencing) of the $CD200_{C\text{-}tail}$. Subsequently there occurs an altered expression of a limited number of genes, many of which are transcription factors (TFs) known to be associated with regulation of cell proliferation. Altered expression of these TFs was also prominent following transfection of $CD200^+$ B cell lines and fresh patient CLL cells with a vector construct containing the $CD200_{C\text{-}tail}$. Artificial transfection of non $CD200^+$ Hek293 cells with this $CD200_{C\text{-}tail}$ construct resulted in altered expression of most of these same genes. Introduction of a siRNA for one of these TFs, POTEA, reversed $CD200_{C\text{-}tail}$ regulation of altered cell proliferation.

1. Introduction

CD200 engagement of its cognate receptor, CD200R, has been shown to cause attenuation of both inflammatory responses and the development of acquired immune responses, as determined by, amongst others, a suppression of T cell mediated transplant rejection response and inflammatory cytokine production in models of autoimmune arthritis [1]. While in mice a number of different CD200Rs have been described, in humans it seems only one, conventionally referred to as CD200R1, is transcribed and biologically functional [1]. Since the C-tail of CD200 has no consensus signaling motifs, or consensus sites for binding adapter molecules which might act as signaling agents, it was widely assumed that only the extracellular region of CD200 (CD200v + c) was of importance in signaling in a cellular milieu. Indeed we recently reported that cleavage of the extracellular region of membrane bound CD200 by extracellular sheddases and/or metalloproteases resulted in production of a soluble form of CD200, sCD200, able to signal through the cognate receptor CD200R on other cells [2]. sCD200 was present in systemic form in normal human serum/plasma [2]. Detectable levels of sCD200 are known to be produced constitutively by CLL cells in vitro and at enhanced levels following PMA stimulation, and levels are elevated in patients with chronic lymphocytic leukemia (CLL) [3]. sCD200 contributes to increased growth and survival of CLL cells as demonstrated in a $NOD.SCID^{IL2r-/-}$ model of the disease [3].

More recently it has become apparent that many Type I membrane bound molecules also undergo intracellular cleavage, by so-called gamma secretase enzymes, with the cytoplasmic tail of these molecules subsequently translocating to the nucleus where they have been shown to alter gene transcription [4]. Thus Notch signaling, a known key step in human cancer growth, has been shown to be modified by gamma-secretase inhibitors which prevent generation of the intracellular (oncogenic) domain of Notch molecules [5]. In a similar fashion, amyloid precursor protein, a large integral membrane protein and known substrate of gamma secretase, is cleaved by both gamma and beta secretases to produce the short 39–42 amino acid peptide, amyloid beta. The gamma secretase cleaved moiety has in turn been shown to form a complex with the nuclear adaptor protein Fe65 and the histone acetyltransferase Tip60 to stimulate transcription [6]. Abnormally folded fibrillar forms of amyloid beta represent the primary component of the

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amyloid plaques found in the brains of Alzheimer's disease patients. Hence there is great interest in development of antagonists to gamma secretase in both cancer and Alzheimer's disease research. Inhibitors of γ-secretase cleavage were reported to prevent cell growth inhibition by the ErbB-4 transmembrane receptor tyrosine kinase, which is dependent upon release of the intracellular domain of ErbB-4 from the membrane and its translocation to the nucleus [7].

We speculated that cleavage of membrane bound CD200 may also involve both extracellular and intracellular events, and explored mechanisms which might identify such a process, and its outcome(s). We show below that in cells actively shedding CD200 an intracytoplasmic form(s) is produced ($CD200_{C\text{-}tail}$) which binds to nuclear DNA at a limited number of sites, identified by Chip sequencing. These sites in turn represent regions encoding TFs, many of which are implicated in regulation of cell proliferation. Transfection of cells by an artificial construct containing $CD200_{C\text{-}tail}$ produces changes in many of these same TFs, and we show that attenuation of expression of at least one of these alters proliferation of CLL cells.

2. Materials and methods

2.1. Cells, reagents, antibodies and ELISA for sCD200

Peripheral blood from treatment naïve CLL patients were collected at routine follow-up visits and $CD19^+CD5^+$ CLL cells purified using the Rosette Sep human B cell enrichment cocktail (StemCell Technologies, Vancouver, BC) as described previously [3]. All samples were from subjects with an un-mutated IgHV who had been untreated for at least 3 months before the peripheral blood was collected. A total of 18 individual subjects were used in the data described below, with all CLLs contributing to analysis spontaneously releasing detectable (> 1 ng/ml) sCD200 into the culture supernatant over a 24hr period. We showed previously that different CLL samples show high/low sCD200 release independent of membrane bound levels of CD200 expression [2,3]. Purified CLL cells were cultured in AIMV medium (Invitrogen, Carlsbad, CA) supplemented with 5×10^{-6} M β-mercaptoethanol (2-ME) (Sigma) and supernatants of cultures were assayed for release of sCD200 by ELISA, using capture and detection anti-CD200 antibodies as described elsewhere [3]. Where indicated, cells were stimulated with Phorbol 12-myristate 13-acetate (PMA) purchased from Sigma-Aldrich at a concentration of 40 ng/ml. The protease inhibitors and γ-secretase inhibitors, GM6001 and DAPT respectively, were purchased from Calbiochem and used as indicated in the experiments described.

The monoclonal rat anti-hCD200 antibodies 1B9, and the polyclonal rabbit serum against the extracellular region of CD200 (CD200v + c), were described previously [3]. A polyclonal rabbit serum against the cytoplasmic region of human CD200 was prepared as described earlier, following immunization against a peptide containing the 19-amino acids of the carboxyl-terminal domain of human CD200 (*KRHRNQDRGELSQGVQKMT*), synthesized at the Hospital for Sick Children (Supplementary Fig. 1). Confirmation of the specificity for the cytoplasmic domain of CD200 ($CD200_{C\text{-}tail}$) came from studies testing the serum with cell lysates from Hek-hCD200 transfected cells or cells expressing only the extracellular domain of CD200 on Western blots, as described elsewhere [2].

Chromatin immunoprecipitation (Chip) assays were performed using a commercial kit (Millipore Sigma, Etobicoke, Canada) [8]. Protein-protein and protein-DNA were cross-linked (by formaldehyde) in CLL cells documented in independent studies to shed sCD200. After cross-linking, cells were lysed and crude extracts sonicated to shear the DNA. Proteins along with cross-linked DNA were subsequently immunoprecipitated with polyclonal antibody to cytoplasmic region of hCD200, the complex captured using Protein A beads, and samples analysed by ChiP-Westerns on PAGE-gels. Protein-DNA cross-links in the immunoprecipitated material were reversed, and DNA fragments were purified, PCR amplified and sequenced (ChiP-seq) in the University Health Network facility (Supplementary (Excel) Table 1). The software used for analysis were “ChipSeq Peak Finder” and “FindPeaks”. Documentation of the PCR primers for qPCR amplification of TFs identified by ChiP-seq is shown in Supplementary Table 2 (see 9–14).

2.2. Transfection of CLL cells with human-gene specific POTEA siRNAs

3 Unique 27 mer siRNA duplexes (SR317504A; SR317504B; SR317504C) were purchased from Origene, along with a negative control silencer (SR3004). 2×10^6 fresh CLL cells were used per transfection, with all transfections performed in triplicate. Cells were transfected using Lipofectamine siRNA complex (made in OptiMEM). 5hr post transfection an equal volume of AIMV medium was added to all cells and incubation continued for 24 and 48hr at 37 °C. Cell viability was measured (Trypan blue exclusion) at 24/48 h and recorded for both control cells, and those receiving control vs gene-specific silencers. In addition, qPCR was performed on cells at 48 h to assess the degree of inhibition of expression of POTEA expression.

2.3. Construction of expression vector containing $CD200_{C\text{-}tail}$ linked to V5 marker ($CD200_{C\text{-}tail\text{-}V5}$)

A construct encoding human $CD200_{C\text{-}tail\text{-}V5}$ (Supplementary Fig. 1) was inserted into a commercial pIRES neo 3.0 vector using Age1 and Not1 sites. Hek cells (known not to express human CD200) were transfected with this vector using Lipofectamine 2000 (Thermo Fisher Scientific, Mississauga, Canada) in a culture chamber slide with 5×10^5 Hek cells.

After 48hr an aliquot of cells was fixed with 1%PFA for 15 min at room temperature, permeabilized with 0.5% Triton X-100 for 20 min at room temperature, blocked with goat serum (30 min at room temperature) washed and stained with mouse anti-V5 antibody (Thermo Fisher Scientific, Mississauga, Canada). Following washing, AlexaFluor 488 goat anti-mouse antibody (Abcam, Toronto, Canada) was added for 30 min at room temperature in the dark, the slide washed thoroughly, DAPI added, and the covered slide examined under confocal microscopy.

A further aliquot of the same transfected cells was used for extraction of mRNA for qPCR analysis of TF gene expression.

3. Results

3.1. ChiP-Western identifies cytoplasmic domain of CD200 in PMA-stimulated CLL cells shedding sCD200

We have shown earlier that sCD200 release from CLL cells, was enhanced several fold following PMA stimulation [2,3]. To explore whether extracellular release of sCD200 was simultaneously accompanied by intracellular translocation of a cytoplasmic region of CD200, we performed the following studies. Firstly, CLL cells were cultured in AIMV medium with/without PMA stimulation and concentrated cell supernatants, or cytoplasmic and cell membrane extracts analysed on Western gels with rabbit antibodies directed to the extracellular domains of CD200 (V + C) or the isolated cytoplasmic domains ($CD200_{C\text{-}tail}$). Data shown in Fig. 1A/B show that PMA stimulated cells shed into the supernatant a molecule (~45 kDa) detected by an antibody to the extracellular domain of CD200, which lacks determinants detected by antibody to the C-tail of CD200 (Fig. 2B). In contrast, isolated cytoplasmic extracts of these same cells, but not membrane extracts, contain a low molecular eight molecules (~6–8 kDa) which is detected by an antibody to this cytoplasmic tail region (**1C**), but not antibody to the extracellular domain (**1D**). Confirmation of the restricted specificity of these two antibodies was reported elsewhere [2].

In follow-up studies (Fig. 2), again only in cells with significant detectable sCD200 release (Fig. 2A) was there evidence for a band in Western gels, detected by rabbit antibody to the cytoplasmic region of