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Short communication

Upregulation of integrin expression on monocytes in multiple sclerosis patients treated with natalizumab

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ABSTRACT

Natalizumab is a humanized monoclonal antibody against the α4 subunit of VLA-4 integrin that is used to treat conditions such as multiple sclerosis (MS). Although its effects on lymphocytes have been widely described, little is known about its effects on monocytes. Here we described the effects of natalizumab treatment on peripheral blood monocytes from a small cohort of MS patients in terms of relative frequencies and surface integrin (CD49d and CD18) expression. We showed that natalizumab treatment altered the surface integrin expression on monocyte subsets in the peripheral compartment, suggesting a role for them as mediators of natalizumab effects.

1. Introduction

Multiple sclerosis (MS) is an acquired autoimmune disease leading to extensive and chronic neurodegeneration as a consequence of the damage to myelin (Compston & Coles, 2008). Worldwide, there are 1.3 million people with MS, 400,000 of whom are in Europe, with a prevalence ranging between 2 and 150 per 100,000 individuals (Compston & Coles, 2008).

Natalizumab is a humanized monoclonal antibody used for MS and other inflammatory disorder therapy (Léger et al., 1997). It binds the α_4 chain, also known as CD49d, of the $\alpha_4\beta_1$ (very late antigen 4, VLA-4) and $\alpha_4\beta_7$ integrins, which are present on the surface of the leukocytes except neutrophils and are involved in their egress from the bloodstream (Muller, 2011). The activity of natalizumab causes a reduced leukocyte extravasation into peripheral tissues, decreasing the inflammatory process (Stüve & Bennett, 2007). Natalizumab increases the number of circulating leukocytes (Planas et al., 2012); mobilizes hematopoietic stem cells from bone marrow (Bonig et al., 2008; Zohren et al., 2008), and disproportionally increases the number of circulating pre-B and B cells (Krumbholz et al., 2008). Stüve et al. (2006) showed that the natalizumab treatment leads to an alteration of the $CD4^+/CD8^+$ T cell ratio in the cerebrospinal fluid (CSF), as occurs in HIV-positive patients. This finding is of interest considering that progressive multifocal leukoencephalopathy (PML), a demyelinating disease caused by polyomavirus JC (JCV), is the most serious adverse effect of natalizumab therapy (Stüve et al., 2007). Efforts have been made to understand how this treatment may induce JCV reactivation, but the mechanism is not completely understood (Schwab et al., 2013). Moreover, peculiar alteration within the monocyte compartment in the peripheral blood during the PML phase, in an MS patient treated with natalizumab (Schwab et al., 2012), and the significant increase of the $CD14^+$ monocyte percentage in the CSF of natalizumab-treated patients compared with naïve MS patients have been described (Schneider-Hohendorf et al., 2014). Circulating monocytes were recently described as one of the leukocyte populations harboring more frequently JCV DNA in MS patients (Chalkias et al., 2014). Despite these reports, the effects of natalizumab treatment on the monocyte population have been poorly investigated.

We followed natalizumab-treated MS patients for one year, from the time they started the therapy, to evaluate whether the treatment induced alterations in the circulating monocytes and their subsets, identified as classical $CD14^+CD16^-$, intermediate $CD14^+CD16^+$ and non-classical $CD14^{dim}CD16^+$. Surface expression levels of CD49d and CD18 (integrin subunit β_2), the beta chain of different integrins important for monocyte extravasation (Muller, 2011), were evaluated.

2. Materials and methods

2.1. Patients

After obtaining signed informed consents based on the local ethics committee guidelines, 11 patients with clinically defined relapsing–remitting MS and treated with natalizumab (MSN) were recruited at

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Istituto Neurologico Mondino (Pavia, Italy). Patients received the dose of 300 mg of natalizumab over a one-hour intravenous infusion every four weeks. Peripheral blood was collected immediately before the first (T0) and the thirteen infusions (T12). Demographic and clinical characteristics of the patients are described in Table 1. MRI analysis performed at T0 and at T12 revealed no change in number or activity of the lesions. Eleven healthy subjects (HS) were enrolled as control group, and peripheral blood was collected once.

2.2. Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Paque PLUS (GE Healthcare) and cryopreserved in freezing media (90% fetal bovine serum, 10% DMSO).

2.3. Flow cytometry analysis

PBMC surface 4-color staining was performed in a staining buffer (PBS + 2% FBS) for 30 min at 4 °C in the dark with the appropriate combinations of saturating concentrations of the conjugated monoclonal antibodies (BD Biosciences): CD45-PerCP, CD14-APC, CD16-PE, CD18-FITC, CD49d-FITC and isotype $IgG1_k$-FITC. For flow cytometry analysis, BD FACSCalibur™ and the BD FACSDiva™ Software were employed.

2.4. Statistical methods

Statistical differences between groups or intra-individual changes over time were determined by unpaired (T0/T12 vs HS) or paired (T0 vs T12) t-test, or Wilcoxon and Mann–Whitney U-value non parametric test when the tested parameter was not normally distributed. Mean ± 95% CI were used to describe the results (GraphPad Prism software, La Jolla, CA). A p value of <0.05 was considered to be statistically significant.

3. Results

3.1. Leukocyte subset relative frequencies

The monocyte subset relative frequencies were analyzed over time to highlight any variations that could be caused by natalizumab treatment. We detected a decrease in the relative frequency of total monocytes at T12 (14.50 ± 6.57) compared with T0 (32.35 ± 9.73) (Fig. 1A). This result was further investigated by studying the monocyte subsets ($CD14^+CD16^-$, $CD14^+CD16^+$ and $CD14^{dim}CD16^+$), but no difference was observed (Fig. 1B)., The patients' distribution of monocyte subsets differed from that of HS: the proportion of $CD14^+CD16^-$ was lower (T0: 75.91 ± 4.54, T12: 59.66 ± 17.04, HS: 88.90 ± 1.93), whereas those of both $CD14^+CD16^+$ (T0: 13.99 ± 3.09, T12: 30.88 ± 18.65, HS: 6.36 ± 0.85) and $CD14^{dim}CD16^+$ (T0: 10.10 ± 3.03, T12: 9.46 ± 2.91, HS: 4.73 ± 1.46) were higher.

3.2. Integrin expression

The surface expression of the integrin subunit CD49d was assessed using an antibody that does not compete for binding with natalizumab (Defer et al., 2012). When analyzing the total CD49d expression over time, $CD14^+CD16^+$ and $CD14^{dim}CD16^+$ showed a higher surface expression of CD49d after one year of treatment (T0: 1.65 ± 0.32 and 2.09 ± 0.48, T12: 2.39 ± 0.74 and 3.14 ± 1.02, Fig. 2A, B); however, no difference compared with HS was observed (2.34 ± 0.69 and 2.95 ± 0.73 Fig. 2A, B).

$CD14^+CD16^+$ and $CD14^{dim}CD16^+$ monocytes showed differences in their CD18 surface expression, which increased after natalizumab treatment (T0: 24.51 ± 6.81 and 23.71 ± 6.38, T12: 52.45 ± 22.53 and 46.71 ± 14.91, Fig. 2C, D). The level of CD18 on $CD14^{dim}CD16^+$ at T12 was higher than in HS (28.73 ± 13.21, Fig. 2C, D).

4. Discussion

We followed 11 MS patients for 12 months and evaluated immunological changes in the peripheral blood. We focused on the monocyte population and its subsets, defined as classical $CD14^+CD16^-$, intermediate $CD14^+CD16^+$ and non-classical $CD14^{dim}CD16^+$, since monocytes are second only to $CD34^+$ cells in terms of frequency of cells harboring JCV DNA (Chalkias et al., 2014). Additionally, natalizumab treatment greatly increases the percentage of monocytes in CSF (Schneider-Hohendorf et al., 2014).

Reduced proportion of circulating monocytes was observed, as already described (Skarica et al., 2011): this decrease involved evenly all the monocyte subsets, suggesting a smaller growth in terms of absolute number of monocytes compared with the other leukocyte subsets, rather than an impairment in the viability of any monocyte subset.

A monoclonal antibody not competing with natalizumab was used to analyze the surface expression of both bound and unbound CD49d (Defer et al., 2012). A consistent increase of total CD49d expression on the surface of $CD14^+CD16^+$ and $CD14^{dim}CD16^+$ cells after 12 months of therapy was detected. To this regard, the short life of monocytes, about one to three days, could have prevented them to undergo the natalizumab long-lasting effects.

LFA1 (composed by the integrin subunits α_L, or CD11a, and β_2, or CD18) and MAC1 (α_M, or CD11b, and CD18) are involved in the transendothelial migration of leukocytes (Muller, 2011) playing a more relevant role in monocyte adhesion/transmigration than VLA-4. A blockade of either simultaneously CD11a and CD11b or only CD18 showed that it impaired the monocyte ability to transmigrate across an in vitro cell monolayer, with the strongest impairment observed after the CD18 blockade. No defect was detected after the individual blockade of CD11a or CD11b, likely due to a redundant role of LFA1 and MAC1. Conversely, blocking CD29 only slightly reduced the monocyte transmigration ability. In our study, an increase in the surface level of CD18 on $CD14^+CD16^+$ and $CD14^{dim}CD16^+$ monocyte subsets after 12 months of therapy was shown. It is well known that natalizumab binds to CD49d, that is involved in the extravasation process, activating some signaling pathways into the cells, such as the MAPK/ERK pathway (Benkert et al., 2012). The activation status induced by the interaction natalizumab/CD49d could lead to the increased surface expression of CD18 (Fougerolles & Koteliansky, 2002).

It could be speculated that the significant rise of CD49d and CD18 surface expression on $CD14^+CD16^+$, $CD14^{dim}CD16^+$ and the slight rise on $CD14^+CD16^-$ monocytes, could account for the increased extravasation ability and the resulting increase of the monocyte frequency in the CSF of natalizumab-treated MS patients (Schneider-Hohendorf et al., 2014). However, it should be considered that the ability of monocytes to pass through the BBB also depends on several other factors. Since monocytes are one of the subsets harboring the highest levels of JCV DNA (Chalkias et al., 2014), JCV might also exploit these immune

Table 1
Clinical and demographic characteristics of the patients.

		N° patients	Age-years (±sd)	M/F	Disease duration-years (±sd)	EDSS (range)	No. of patients with relapses
MSN	T0	11	37.8 (±9.2)	3/8	12.7 (±7.3)	1.5 (0–4.5)	2
	T12	11	38.8 (±9.1)	3/8	13.7 (±8.3)	1.9 (0–6.5)	0
HS		11	40.3 (±8.2)	2/9	/	/	/