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Immune markers in the RASopathy neurofibromatosis type 1

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ABSTRACT

Neurofibromatosis type 1 (NF1) is a genetic disorder with an early mortality determined mostly by malignancy. Little is known about the immunosurveillance factors in NF1 patients. In this study we evaluated inflammatory markers and their cellular sources in NF1 patients to try understanding the relation of immune factors and the tumorigenesis that characterizes the disease. Using flow cytometry and ELISA, we assayed cytokines, co-stimulatory molecules, the functional state of circulating blood cells and cytokine plasma levels in a case–control transversal study. The frequency of CD4+ T cells seems reduced. In addition, a shift towards an anti-inflammatory profile was observed in cells expressing cytokines, except for a small subpopulation of CD8+ T cells that displayed an increased frequency of cells expressing the pro-inflammatory cytokine Tumor necrosis factor (TNF-α), while plasma soluble levels of Transforming growth factor-beta (TGF-β) and interleukin-6 (IL-6) were increased in NF1 patients. Knowledge of the regulation of NF1 and the role of TGF-beta signaling pathway in malignant peripheral nerve sheath tumor pathogenesis might shed light on molecular carcinogenesis mechanisms and lead to putative interventions both in prevention and treatment of malignant tumors.

1. Introduction

Neurofibromatosis type 1 (NF1) is part of the RASopathies group of syndromes, caused by mutations in genes of the Ras-MAPK pathways. It is a chronic, progressive and autosomal dominant disorder with a prevalence of 1:3500 individuals. NF1 is caused by inherited or de novo mutations in the *neurofibromin* gene (chromosome 17 — 17q11.2 region), resulting in dysfunction of Ras, a key tumor suppressor gene (Viskochil et al., 1990; White et al., 1991). The neurofibromin GTPase-activating domain promotes Ras inactivation (DeClue et al., 1991) thus, its dysfunction leads to overactivation of Ras and of the downstream Raf-MAP kinase pathway, resulting in favorable conditions to cell proliferation (Gottfried et al., 2010).

The most frequent NF1 clinical phenotype includes *café-au-lait* macules and benign neurofibromas which are commonly accompanied by cognitive and skeletal disorders, axillary or inguinal ephelides and Lisch nodules (Gutmann et al., 1997; Gottfried et al., 2006). Plexiform neurofibromas (PNFs), occurring in 30–50% of NF1 patients, constitute an important source of morbidity (Laycock-van Spyk et al., 2011). Although not all NF1 patients have severe complications or esthetic deformations, NF1 has a progressive and unpredictable course (Riccardi, 1999). Connective and soft tissue neoplasms were found to be responsible for a decrease of 8–15 years in life expectancy in NF1 patients (Rasmussen et al., 2001; Evans et al., 2011). NF1 individuals with pre-existing and internal PNFs have a 20-fold higher risk to courses with an MPNST than NF1 without such tumors (Tucker et al., 2005). Longitudinal studies evaluating relative risk of malignization in English and Swedish NF1 population (Sørensen et al., 1986; Zöller et al., 1995; Walker et al., 2006) observed an relative risk estimate between 2.7 and 4 of higher risk of malignancy, specially in individuals with less than 50 years, and with tumors from CNS, connective tissues and breast. This risk makes NF1 the most common genetic disorder associated with malignancy. In addition, neurofibromin is mutated in 7% of individuals with acute myelogenous leukemia (Hope and Mulvihill, 1981; Gottfried et al., 2010) and is associated to gliomas, not only with the NF1-related tumors but also in 15% of sporadic cases (McGillicuddy et al., 2009; Gottfried et al., 2010).

Immuno-oncology research has provided evidence that the immune system recognizes tumor cells and is able to prevent their development

Abbreviations: APC, Allophycocyanin; IL-1β, interleukin-1 beta; IL-6, interleukin-6; IL-8, interleukin-8; IL-10, interleukin-10; IL-17, interleukin-17; INF, interferon; IP-10, interferon-gamma inducible; GTP, guanosine triphosphate; MW, Mann–Whitney; MCP-1, monocyte chemoattractant protein-1; NF1, neurofibromatosis type 1; MPNST, malignant peripheral nerve sheath tumor; PBMCs, peripheral blood mononuclear cells; PE, phycoerythrin; PNF, plexiform neurofibroma; MAPK, MAP-kinase; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha; STNF RI and STNF RII, tumor necrosis factor-alpha receptors type I and type II.

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and progression. In cases of malignant progression, immunosuppression that determines the antitumor response and effectiveness of the treatment can be commonly found (Finn, 2012). The understanding of cancer is being expanded from knowledge about cancer cells to the comprehension of the host conditions and microenvironments that favor malignant growth. Interest in neurofibromatosis immunology was granted by the recognition of NF1 as a useful biological model to study overactivation of the Ras pathway, which is involved in ~20–30% of human tumors (Forbes et al., 2011; Prior et al., 2012). Thus, NF1 constitutes a valuable condition to investigate cancer biology, in a predisposing microenvironment and monogenic disease context, where only one known mutated gene per se predisposes to different forms of malignancy (Hollstein and Cichowski, 2013).

Despite intense research efforts, a therapeutic grip on Ras-associated tumorigenesis mechanisms has remained elusive. Therefore, the study of peripheral inflammatory factors might be important in identifying biomarkers of the pathophysiological processes involved in NF1-associated malignization, leading to improved diagnosis and enabling the development of new therapeutic strategies, such as immunotherapy.

The fact that immune function and cell distribution has not been studied in NF1 patients makes it an interesting topic. Therefore, in this study, we have assayed the expression of cytokines as well as important co-stimulatory molecules in immune blood cells and plasma soluble levels of cytokines in healthy controls and NF1 patients. In addition, the distribution of T and B lymphocytes, monocytes and NK cells were evaluated. We have tried to obtain a profile of immune cells distribution and molecules related to immune system in patients with NF1 RASopathy.

2. Patients and methods

2.1. Subjects

Twenty-five NF1 patients of both sexes were enrolled from the Outpatient Neurofibromatosis Reference Center of Minas Gerais, Brazil, where most patients have at least an annual follow-up. Nineteen healthy volunteers of both sexes formed the paired control group. This study was approved by the Ethics Committee of Universidade Federal de Minas Gerais—Brazil, CAAE 01344212.2.0000.5149/CEP 36675. All subjects (or legal guardian) gave their informed consent to participating in the study, which was conducted in accordance with the provisions of the Helsinki Declaration. At enrollment, NF1 patients did not have known malignancies and they were followed for three years.

2.2. Cell cultures

Peripheral blood mononuclear cells (PBMCs) were prepared from approximately 9 mL of venous peripheral blood collected in heparinized tubes. PBMCs were separated using a Ficoll/Hypaque (Sigma) gradient and cells (1.5×10^5) were cultured in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated human serum (Sigma), 1 mM L-glutamine and 200 U of penicillin (Sigma). Cultures were harvested after 18 h.

2.3. Flow cytometry

Flow cytometric analysis was performed as previously described by Torres et al. (2009). In brief, PBMCs (1.5×10^5) were cultured in 200 μL of media in 96 well plates for 18 h were with added brefeldin A (1 μL/mL) (Sigma) during the last 4 h to inhibit protein secretion by the Golgi complex. Then, cells were stained with fluorescein isothiocyanate (FITC), CyChrome 5 (cy5), CyChrome 7 (cy7), BD Horizon V450, BD Horizon V500 and Allophycocyanin (APC). PBMCs were washed with 0.1% sodium azide PBS (GIBCO, Life Technologies) and fixed with 2% formaldehyde (Sigma) in PBS. The antibodies used for extracellular staining were anti-CD4-V500, anti-CD8-APC, anti-CD14-V450, anti-CD69-FITC, anti-HLADR-cy7, anti-CD95-FITC, anti-CD95L-PE, anti-CTLA4-cy5, anti-CD40L-cy5, anti-CD80-cy5 and anti-CD86-APC (BD Pharmingen). After extracellular staining, the cells were permeabilized with a solution of 0.5% saponin (Sigma) and stained for cytoplasmic cytokines, for 30 min at room temperature, using anti-cytokine monoclonal antibodies conjugated with phycoerythrin (PE). For single-cell cytoplasmic cytokine staining, the monoclonal antibodies used were anti-IL-6, anti-IL-10, anti-IFN-gamma, anti-TGF-beta, anti-IL-17A, anti-IL-4, anti-IL-9, anti-IL-1beta and anti-TNF-alpha. FITC-, cy5-, cy7-, PE-, APC-, V450- and V500-labeled immunoglobulin isotype control antibodies were included in all experiments (BD Pharmingen). The stained PBMC samples were acquired using a FACSCanto II flow cytometer (BD) and analyzed using FlowJo software 7.5 (Tree Star), according to the gate strategy shown in Fig. 1.

2.3.1. ELISA

Blood was aseptically collected. Plasma samples were separated and stored at −80 °C until required. Prior to analysis, samples were thawed. The concentrations of TNF-alpha, IL-1beta, IL-10, IL-6, TGF-beta, IL-8, MCP-1, IP-10, and TNF receptors (STNF RI and STNF RII) in the plasma of NF1 patients and controls were measured using sandwich ELISA kits (Duo Set, R&D Systems, Minneapolis, MN, USA). All samples were assayed in duplicate in a single assay. Our intra-assay variation for the ELISA measurements was less than 3%.

2.3.2. Data analysis

Leukocytes and monocytes labeling were analyzed using the FlowJo 7.5 software. Statistical analysis was performed by the GraphPad Prism software, release 5.0 (GraphPad Software, San Diego, CA). Normality distribution of variables was evaluated with the Shapiro–Wilk Test. When results had a normal distribution, unpaired t test was used to compare NF1 patients and healthy controls, and when results showed a non-Gaussian distribution, the Mann–Whitney (MW) nonparametric test was used for comparisons between the two groups. All data are presented as the mean ± standard deviation for normally distributed data or as the median ± range for nonparametric data, as indicated in all cases. p values smaller than or equal to 0.05 were considered to be significant, as indicated in figure legends.

3. Results

Analysis of demographic data indicated no significant difference between control and NF1 groups regarding age ($p = 0.36$) and gender ($p = 0.37$). Approximately 70% of NF1 patients presented plexiform neurofibroma, 20% showed the spinal form, 80% had complaints of pain on plexiform neurofibroma what motivated the following to the reference center and 12% were diagnosed with the MPNST in subsequent three years of follow-up (Table 1).

To analyze the measured immunological data, we initially evaluated the frequency of the following cell types: CD19+ B lymphocytes, CD4+ and CD8+ T cells, CD14+/CD16+ monocytes and CD56+ NK cells as well as T lymphocytes and CD14+ monocytes expressing cytokines and co-stimulatory activation markers. The pro-inflammatory cytokines assayed included INF-gamma, TNF-alpha, IL-17 and IL-6 and the Th2/anti-inflammatory panel included IL-4, IL-10 and TGF-beta. In addition, the functional status of T lymphocytes was measured through the surface markers CD69, HLADR, CD40L, Fas (CD95), Fas ligand (CD95L), cytotoxic t-lymphocyte antigen-4 (CTLA-4), and the functional status of monocytes was measured using CD80, CD86 and HLADR.

Initially, the frequency of immune cells was analyzed in NF1 patients and healthy controls. No differences were found between groups in their frequencies of CD8+ T cells, CD56+ NK cells, CD14+ monocytes and CD14+/CD16+ monocytes (data not shown). In addition, no significant difference was found in the frequency of CD19+ B lymphocytes, however, there was a very significant decrease in CD19+ B cells expressing CD5+ in NF1 patients (11 ± 13% compared with controls 28.0 ± 12%, $p < 0.0001$; Fig. 1H). A less significant decrease in CD4+ T cells was seen the PBMCs from NF1 patients (38 ± 13%), compared