Primary central nervous system lymphoma secretes monocyte chemoattractant protein 1

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Abstract The majority of primary central nervous system lymphomas (PCNSL) are diffuse large B-cell lymphomas. Histologically, reactive T lymphocytes and monohistiocytic cells are found within PCNSL tissue. To clarify the mechanisms of the cellular infiltration, the presence of monocyte chemoattractant protein (MCP-1) was investigated in biopsy samples of 19 cases of PCNSL by means of immunohistochemical staining, double staining with a confocal laser microscope, and Western blot analysis. MCP-1 expression was observed in all PCNSL immunohistochemically. Western blot analysis showed that the concentration of MCP-1 in PCNSL was as high as that in a metastatic brain tumor. In normal brain tissue, MCP-1 was not detected. Confocal laser microscope revealed MCP-1 signals were present in the cells with CD20, a B-cell marker. We concluded that lymphoma cells produced MCP-1, which is an additional cytokine involved in the pathogenesis of PCNSL.

Key words Primary CNS lymphoma · Chemokine · MCP-1

Introduction

The age-adjusted incidence of primary central nervous system lymphoma (PCNSL) has increased since the 1970s as immunocompetent patients with human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome have been increasing in number.[1]

Stereotactic biopsy or tumor resection is the method of choice in establishing the definite diagnosis detecting the lymphoid blasts. The overwhelming majority of PCNSL is represented by diffuse large B-cell lymphoma.[2] Besides neoplastic cells, small reactive T lymphocytes and monohistiocytic cells/activated "microglia" are usually found within and at the periphery of PCNSL foci.[2] Surprisingly, there are reported cases in which many infiltrated monocytes or T lymphocytes overwhelmed the neoplastic lymphoma cells.[2-4]

The mechanism of the recruitment of monocytes in PCNSL is unknown. Monocyte chemoattractant protein 1 (MCP-1), also known as CC chemokine ligand 2 (CCL2), is a small, inducible protein with chemotactic activity and has been considered as an important mediator that specifically stimulates directional migration of T cells and monocytes/macrophages.[5-9]

In this study, we investigated the presence of MCP-1 in PCNSL tissue biopsies and what type of cells produced MCP-1.

Materials and methods

Tissue samples

Nineteen formalin-fixed, paraffin-embedded PCNSL specimens were collected from the Department of Neurosurgery at Fukui University Hospital. Three frozen tissue samples were available for Western blotting analysis. The age of the patients ranged from 50 to 82 years (average, 65.6 years); 9 were men and 10 were women (Table 1).

According to the WHO classification of brain tumors, PCNSL is defined as malignant lymphoma arising in the central nervous system (CNS) in the absence of obvious lymphoma outside the nervous system at the time of diagnosis.[2] Detailed histopathological diagnoses were reviewed with author R.K., senior author T.K., and pathologist Y.I.. For Western blot analysis, a fresh autopsied brain without

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Table 1. Summary of the 19 primary central nervous system lymphoma (PCNSL) patients studied

Case	Age	Sex	Location	Classification	CD68	MCP-1
1	57	M	Bil frontal	B-cell type	+	++
2	62	F	R temporal	B-cell type	+	+
3	50	M	L occipital, bil temporal	B-cell type	+	+
4	63	F	R temporal, cerebellum	B-cell type	++	+
5	64	M	Cerebellum	B-cell type	+	++
6	62	M	R frontal	B-cell type	+	+
7	67	F	R frontal	B-cell type	++	++
8	58	F	Bil frontal	B-cell type	++	+
9	59	F	R frontal, cerebellum	B-cell type	+	+
10	57	M	L basal ganglia	B-cell type	++	+
11	66	F	L frontal	B-cell type	++	+
12	73	M	R periventricle	B-cell type	++	+
13	66	M	L temporal	B-cell type	+	+
14	82	M	R thalmus	B-cell type	++	++
15	75	F	Bil frontal, cerebellum	B-cell type	++	+
16	82	F	L thalmus	B-cell type	++	+
17	76	F	L parietal	B-cell type	++	+
18	60	M	L frontal	B-cell type	+	+
19	67	F	Bil basal ganglia	B-cell type	++	++

R, right; L, left; Bil, bilateral; CD 68, CD68-positive cells in tumor tissue; MCP-1, monocyte chemoattractant protein 1 immunopositivity for tumor cells

tumor was used. Metastatic brain tumors of a renal cell carcinoma and a lung squamous cell carcinoma were also used.

Immunohistochemistry and confocal laser microscope analysis

Immunohistochemical staining was performed according to the standard manufacturer's protocol. Primary antibodies directed to CD45 (leukocyte common antigen; DAKO M0701 1:100), CD68 (histiocyte marker; DAKO M876, 1:100), CD20 (B-cell marker; DAKO M755 1:100), CD45R0 (T-cell marker; DAKO M0742, 1:100), and MCP-1 (R&D Systems; MAB2791, 1:100) were used. Negative control was a parallel section treated as already described with the omission of primary antibody. The staining procedure was achieved by the Envision plus technique (DAKO, Fukui, Japan). Intensity of MCP-1 immunostaining and the degree of infiltration of CD 68-positive cells were judged as follows: (–), (+), and (++).

The cellular source(s) of MCP-1 were assessed by immunohistochemical staining against distinctive cellular markers with confocal laser microscope. As secondary antibodies, fluorescence isothiocyanate (FITC)-labeled antimouse IgG (TAGO, Burlingame, CA, USA; 1:100) and rhodamin-labeled (TAGO; 1:100) antibodies. For double-staining with two monoclonal antibodies, the cells were first stained with B-cell marker CD20 at a 1:100 dilution, then the MCP-1 antibody was adopted.

Confocal fluorescence images were obtained on a Leica TCS SP (Leica Microsystems, Wetzlar, Germany) confocal system, equipped with an Ar/Kr/He/Ne laser combination. Possible cross-talk between FITC and rhodamin, which could give rise to false-positive colocalization of different signals, was avoided by careful selection of the imaging conditions.

Western blot analysis

Archival frozen sections were solubilized in cell lysis buffer (T-per; Pierce Biotechnology, Rockford, IL, USA). Human tonsil was used as positive control. The lysates were centrifuged at 17 000 *g* for 15 min at 4°C to remove cell debris, and the supernatants were stored at –20°C until use. The protein content of the supernatant was quantified using a protein assay kit (Bio Rad Laboratories, Hercules, CA, USA). Aliquots of protein (10 µg) were subjected to Western blot analysis for MCP-1. After electrophoresis on 10%–20% polyacrylamide gradient gels (Real gels plate; Bio Craft, Tokyo, Japan) and electrophoretic transfer onto PVDF-membranes, Immobilon-P (Millipore, Medford, MA, USA), the membranes were then incubated with a human anti-MCP monoclonal antibody and an anti-GAPDH monoclonal antibody (Chemicon International, Temecula, CA, USA). For visualization of the bands, we used horseradish peroxidase conjugated antimouse immunoglobulin-G antibody (Zymed Laboratories, San Francisco, CA, USA), and then the BLAST: Blotting Amplification System (NEN Life Science Products, Boston, MA, USA).

Results

All surgical specimens and medical records were reviewed. Histological diagnosis of malignant lymphoma was confirmed with each author. In general, the specimen showed compact cellular aggregates with angiocentric infiltrating pattern. Immunohistochemical staining with CD 20 confirmed that all cases presented here were B-cell-type lymphoma (see Table 1). With careful observation for infiltrating cells, CD68 (histiomonocytic marker)-positive cells and

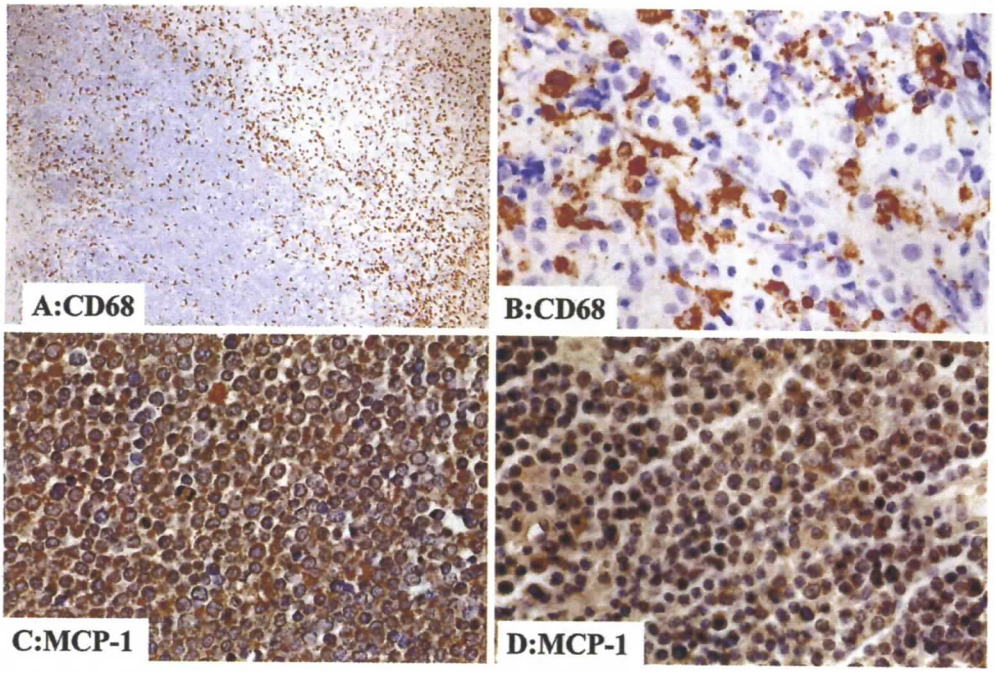

Fig. 1. Case 16 (**A**) and case 17 (**B**). Immunohistochemical staining for CD 68. There were many CD-68-positive cells in lymphoma tissue. Case 5 (**C**) and case 9 (**D**). Lymphoma cells showed a positive reaction for monocyte chemoattractant protein (MCP)-1 antibody. **A** ×100; **B, D** ×400; **C** ×200

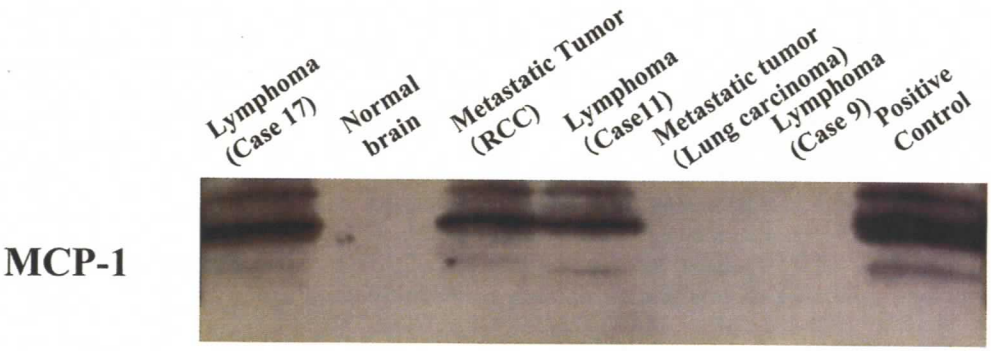

Fig. 2. Western blot analysis for MCP-1. Positive signals were evident in lymphomas, but not in normal brain. *RCC*, renal cell carcinoma

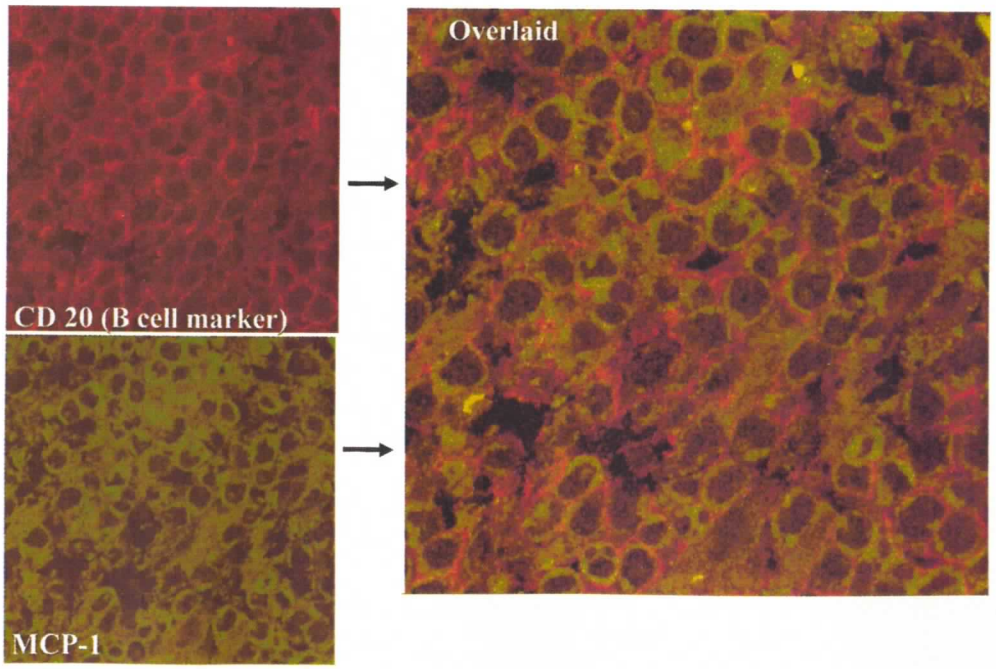

Fig. 3. Case 7. Representative fluorescent immunostained primary central nervous system lymphoma (PCNSL) sections show that *MCP-1* in the cytoplasm [*green*; fluorescence isothiocyanate (FITC)] and *CD20* at the cell surface (*red*; rhodamin). *Overlay image* confirmed MCP-1/CD20 double-positive cells. ×400

CD45R0 (T-cell marker)-positive lymphocytes were scattered in the center of the specimens. In the periphery, CD 68-positive cells were infiltrated predominantly (Fig. 1A,B). CD 68 positive cells were seen in all specimens, but the extent of infiltrated cells differed from case to case. Morphologically, CD 68-positive cells had a larger cytoplasm that was totally different from that of tumor cells (Fig. 1B).

Immunohistochemical staining for MCP-1 revealed that there was cytoplasmic staining of the tumor cells (Fig. 1C,D). All specimens contained MCP-1-positive cells. The intensity of the immunopositivity varied among the cases. Reactive astrocyte-surrounded tumor was very weakly positive for MCP-1 antibody. The presence of MCP-1 was investigated with Western blot analysis (Fig. 2). As PCNSL was usually diagnosed with a small biopsy specimen, there were only three cases for which protein was available. In two cases, MCP-1 protein was abundant in PCNSL by Western blot as well as in metastatic brain tumor. In contrast, there was no significant signal in the normal brain.

To clarify the major cellular sources of MCP-1 in B-cell lymphoma, we investigated double immunofluorescent staining using a confocal laser microscope. As shown in Fig. 3, the signal of MCP-1 (FITC; green) was detected in the cytoplasm of tumor cells. Immunofluoresence of CD20 (rhodamin; red) was positive at the cell surface. Overlaid images with double-staining technique revealed that the MCP-1 signal was concomitant with the cells expressing B-cell marker. We confirmed that lymphoma cells produced MCP-1.

Discussion

The incidence of primary CNS lymphomas has recently increased markedly worldwide as immunocompetent and HIV patients have been increasing.[1] Approximately 98% of PCNSL were B-cell lymphomas with immunohistochemical expression of pan-B markers such as CD20.[2] Besides tumor cells, PCNSL contains many infiltrated microglia/monocytes and mature T cells. We have shown that CD68-positive microglia/monocytes and mature T cells were well recognized by immunohistochemical staining. This fact has been ignored. It was not clear whether tumor-associated CD68-positive cells work to protect brain invasion or to help lymphoma cell proliferation.

Chemokines, i.e., chemotactic cytokines, are small inducible proteins (6–15 kDa) characterized by their capacity to attract subsets of leukocytes,[1,5–13] thereby controlling cell migration during inflammation. More than 20 chemokines have been isolated and described in humans.[5–13] Chemokine production has been investigated in some pathological conditions, including chronic inflammatory diseases,[8,11,14–16] solid tumors,[10,13,17] and systemic malignant lymphomas.[9,18,19]

MCP-1 is a member of the C-C subfamily of chemokines and is typically expressed in tissues during inflammation. MCP-1 is a potent chemoattractant for monocytes and T cells.[6] In PCNSL, there are few studies reported about cytokine production, especially chemokines,[15,20] although systemic lymphoma cells and Hodgkin lymphoma cells produced MCP-1.[9] It was reported that lymph nodes with Hodgkin disease harbored neoplastic cells in a marked lymphocytic infiltration, which were recruited by MCP-1 and other chemokines such as monocyte infiltration protein (MIP)-1-alpha and MIP-1-beta to be highly expressed in neoplastic cells.[21]

Although the proteins were obtained in limited cases (most stored cases were biopsy specimens), Western blot analysis showed that MCP-1 levels were elevated in PCNSL compared with normal brain cells. Immunohistochemical study supported that MCP-1 was detected in all the PCNSL tested. In addition, we confirmed MCP-1 was mainly produced by neoplastic B cells and less frequently by lesional reactive astrocytes. These data implicate MCP-1 in the migration and localization of CD-68-positive monocytes in PCNSL. It is still unknown whether infiltrated monocytes or activated microglia secreted another cytokine or caused cellular interaction in the adjacent brain tissue. They might produce neurotoxic and inflammatory molecules in the tumor site. MCP-1 has various biological activities,[22–24] one of which was brain edema formation by the change of permeability of the blood–brain barrier.[22] MCP-1 also participated in angiogenesis.[23] Abnormal cytokine or chemokine overproduction caused pathophysiological changes and influenced clinical manifestations in PCNSL patients.

In conclusion, MCP-1 expression was found in B-cell lymphoma cells. Additional studies of the potential function of MCP-1 in PCNSL may provide important insights into the pathogenesis of recruitment of inflammatory cells and interstitial reactions of surrounding brain.

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