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Tumorigenesis and Neoplastic Progression

Immunophenotypic Identification and Characterization of Tumor Cells and Infiltrating Cell Populations in Meningiomas

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Meningiomas are primary tumors of the central nervous system composed of both neoplastic and other infiltrating cells. We determined the cellular composition of 51 meningioma samples by multiparameter flow cytometric (MFC) immunophenotyping and investigated the potential relationship between mRNA and protein expression levels of neoplastic cells. For immunophenotypic, morphologic, and cytogenetic characterization of individual cell populations, a large panel of markers was used together with phagocytic/endocytic functional assays and MFC sorting. Overall, our results revealed coexistence of $CD45^-$ neoplastic cells and $CD45^+$ immune infiltrating cells in all meningiomas. Infiltrating cells included tissue macrophages, with an $HLA\text{-}DR^+CD14^+CD45^+CD68^+CD16^{-/+}CD33^{-/+}$ phenotype and high phagocytic/endocytic activity, and a small proportion of cytotoxic lymphocytes (mostly T $CD8^+$ and natural killer cells). Tumor cells expressed multiple cell adhesion proteins, tetraspanins, HLA-I/HLA-DR molecules, complement regulatory proteins, cell surface ectoenzymes, and growth factor receptors. Noteworthy, the relationship between mRNA and protein levels was variable, depending on the proteins evaluated and the level of infiltration by immune cells. In summary, our results indicate that MFC immunophenotyping provides a reliable tool for the characterization of the patterns of protein expression of different cell populations coexisting in meningioma samples, with a more accurate measure of gene expression profiles of tumor cells at the functional/protein level than conventional mRNA microarray, independently of the degree of infiltration of the tumor by immune cells. *(Am J Pathol 2012, 181:1749–1761; http://dx.doi.org/10.1016/j.ajpath.2012.07.033)*

Meningiomas are primary tumors of the central nervous system derived from the meningeal coverings of the spinal cord and the brain.[1] Although the tumor itself is mainly composed of neoplastic cells, the presence of infiltrating inflammatory and normal residual/reactive cells (eg, macrophage/microglial cells and lymphocytes), as detected by immunohistochemistry, have long been reported in meningioma tissue specimens.[2–7] Infiltrating inflammatory cells are involved in the pathogenesis of multiple different tumors, where they may be associated with unique clinical behavior.[3,5,8] In turn, their presence may hamper precise evaluation of tumor cell–specific alterations, particularly quantitative assessment of their biochemical and molecular features (eg, RNA or protein expression levels) due to variable numbers of infiltrating inflammatory cells in the sample.[9,10]

Because of these limitations, lately, techniques enabling isolation of individual cell populations from heterogeneous and complex tumor tissues (eg, laser capture microdissection) are more frequently applied. However,

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these strategies do not allow isolation of large numbers of cells, they are not compatible with live cell analyses, and the limited amount of nucleic acids and other cell components obtained from microdissected samples limits their direct use for high-throughput molecular studies, such as microarray-based gene analysis.[11–13] Alternatively, MFC immunophenotyping is a well-suited method for simultaneous identification, characterization, and isolation of different cell populations in a sample, with purified live cells being placed in a single cell suspension. In addition, MFC immunophenotyping allows quantitative evaluation of protein expression levels in large numbers of individual cells, with highly reproducible and statistically reliable results.[14] Consequently, MFC immunophenotyping emerges as an attractive tool for objective evaluation of the cellular composition of tumor samples, assessment of protein expression profiles (PEPs) of both purified tumor and reactive/inflammatory cells, and determination of the clinical impact of such inflammatory infiltrates.

The number of reported MFC immunophenotypic studies of meningiomas is limited, and these studies are typically restricted to the analysis of the expression of a few individual markers for the whole sample cellularity. Among other markers, such studies reported expression of the CD44 cell adhesion molecule related to tumor invasion and metastasis[15,16] and of CD68, a monocyte/macrophage associated marker, which could potentially be expressed by the tumor cells but also by infiltrating inflammatory cells.[4] To the best of our knowledge, no study has been reported so far in which the relationship between the microarray gene expression profiles (GEPs) and the proteins coded by the affected genes has been specifically evaluated for meningioma tumor cells.

We used MFC phenotyping to analyze the cellular composition and phenotype of 51 meningiomas for a broad set of proteins. In a subset of samples, we further evaluated both the impact of freezing on the PEP of tumor cells and the relationship in individual samples between the amount of mRNA and the corresponding protein levels. Overall, our results reveal that meningiomas systematically display infiltration by inflammatory cells (mainly tissue macrophages) among a major but variable percentage of neoplastic cells. The PEP of tumor cells was not significantly affected in frozen versus fresh tumor samples, whereas the relationship between mRNA and protein levels was variable, depending on the specific proteins evaluated.

Materials and Methods

Patients and Samples

A total of 51 patients diagnosed as having meningioma (14 males and 37 females mean ± SD age, 58 ± 13 years; age range, 30 to 84 years) were analyzed; all but 2 patients (96%) underwent complete tumor resection at the Neurosurgery Service of the University Hospital of Salamanca (Salamanca, Spain). Patients were diagnosed as having meningioma and classified according to the World Health Organization criteria.[17] Forty-nine patients (96%) had benign/grade I meningiomas, corresponding to 19 meningothelial meningiomas (37%), 16 transitional (31%), 7 psammomatous (14%), 5 fibroblastic (10%), 1 angiomatous (2%), and 1 secretory meningioma (2%). Eleven of these 49 tumors displayed transitional features among distinct histologic subtypes (predominance of one histologic subtype and local focus of another subtype; Table 1); the other 2 patients (4%) had grade III meningiomas (one papillary and another rhabdoid meningioma; Table 1). Tumor tissue samples were obtained from each patient at diagnostic surgery after informed consent was given; the study was approved by the local ethics committee and institutional review board of the University Hospital of Salamanca. Table 1 summarizes the most relevant clinical, histopathologic, and cytogenetic characteristics of the 51 patients studied, including the frequency and degree of brain edema. None of the tumor samples contained brain tissue, and only one meningioma had invasion into the bone.

Fresh meningioma tumor samples were frozen in liquid nitrogen immediately after surgical removal and stored at −150°C (freshly frozen samples). In a subgroup of 18 of 51 samples, both fresh (processed <4 hours after surgery) and freshly frozen tumor tissue samples were analyzed in parallel by MFC immunophenotyping.

MFC Immunophenotypic Studies

Freshly frozen tumor samples were thawed in RPMI 1640 medium with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C, following conventional procedures. Single tumor cell suspensions were obtained through conventional mechanical disaggregation procedures[18] in PBS containing 10% fetal bovine serum (Invitrogen), 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO), and 2 mmol/L EDTA (Merck, Darmstadt, Germany). Meningioma cells were stained (30 minutes at 4°C, in the darkness) with the following monoclonal antibodies in 3-color combinations, Pacific blue (PacB)/fluorescein isothiocyanate (FITC)/phycoerythrin (PE), which systematically contained the DRAQ5 DNA dye (Cytognos SL, Salamanca, Spain) and CD45-PacB (Dako, Glostrup, Denmark), for reproducible identification of nucleated cells and leukocytes in the sample, respectively: HLA-DR-FITC, CD2-FITC, CD13-PE, CD14-PE, CD33-PE, CD58-PE, CD69-PE, and HER2/neu-PE (all from Becton/Dickinson Biosciences, San Jose, CA); CD22-FITC, CD37-FITC, CD53-PE, CD55-FITC, CD81-PE, CD99-PE, CD200-PE, epidermal growth factor receptor (EGFR)-PE, and insulin-like growth factor receptor (IGFR)-PE (all from BD Pharmigen, San Diego, CA); HLA-I-FITC, CD9-FITC, CD16-FITC, and CD63-FITC purchased from Beckman Coulter (Miami, FL); CD44-PE and CD59-FITC obtained from Immunostep SL (Salamanca, Spain); and CD38-FITC, Bcl2-FITC, and CD68-FITC purchased from Cytognos SL, Dako, and An der Grub (Vienna, Austria), respectively (Table 2). For the specific identification of lymphocyte subsets, an additional 5-color staining was performed: CD45 pacific orange (PacO; Invitrogen), CD3-PacB (BD Pharmingen), CD8-FITC (BD Biosci-

Table 1. Relevant Clinical, Histopathologic, and Genetic Characteristics of the 51 Meningiomas in Which Immunophenotypic Patterns of Tumor Cells Were Analyzed by Flow Cytometry

Tumor no.	Sex	Age, years	Relapse	Histologic subtype (WHO)	Grade	Localization	Edema	FISH karyotype
1	M	41	Yes	Meningothelial (with xanthomatous areas)	I	Convexity	Light (+)	−(1p/6/14/22/)
2	F	30	Yes	Papillary	III	Convexity	Moderate (++)	−14 +(1q/22/X)
3	F	35	No	Meningothelial (with microcystic areas)	I	Convexity/parasagittal	Severe (+++)	−14 +(7/10/15/17) ++18
4	F	76	No	Meningothelial	I	Convexity/parasagittal	No	−(14/22/X)
5	F	60	Yes	Transitional	I	Convexity	Moderate (++)	−(1p/14/22) +9
6	F	52	No	Transitional	I	Convexity/parasagittal	Moderate (++)	−(1/10/14/15/17/18/22/X)
7	F	70	No	Meningothelial	I	Cranial base	Moderate (++)	−(1p/14/22/X) +1q
8	M	56	No	Meningothelial	I	Cranial base	No	D
9	F	54	No	Psammomatous	I	Cranial base	No	−22
10	F	68	No	Transitional	I	Cranial base	Severe (+++)	D
11	F	69	No	Meningothelial	I	Cranial base	Light (+)	D
12	M	54	No	Meningothelial	I	Cranial base	Moderate (++)	D
13	F	34	No	Transitional	I	Convexity	No	−22
14	F	42	No	Meningothelial	I	Tentorial	No	D
15	F	84	No	Meningothelial	I	Convexity	Severe (+++)	−(1p/10/14/22/18)
16	F	53	No	Transitional	I	Convexity	Light (+)	−22
17	F	66	No	Fibroblastic (with transitional areas)	I	Convexity	Moderate (++)	−22
18	F	49	No	Transitional (with xanthomatous areas)	I	Convexity	Light (+)	−22
19	F	42	No	Meningothelial	I	Convexity	Moderate (++)	D
20	M	68	No	Meningothelial	I	Convexity/parasagittal	Moderate (++)	+(1q/7/10/15/17/18/22/Y/X)
21	F	43	No	Meningothelial	I	Convexity	No	D
22	F	42	No	Transitional	I	Convexity/parasagittal	No	−22
23	F	42	No	Transitional	I	Convexity	Light (+)	D
24	F	58	No	Fibroblastic (with transitional areas)	I	Convexity/parasagittal	Severe (+++)	−22
25	F	75	No	Psammomatous (with metaplastic areas)	I	Spinal	No	−22
26	F	78	No	Psammomatous	I	Spinal	No	−22
27	F	61	No	Secretory	I	Cranial base	Moderate (++)	D
28	M	56	No	Rhabdoid	III	Convexity/parasagittal	Severe (+++)	D
29	M	61	No	Meningothelial	I	Convexity	Light (+)	−(14/22/Y)
30	F	56	No	Fibroblastic	I	Convexity/parasagittal	Light (+)	−22
31	F	57	No	Fibroblastic	I	Cranial base	No	−22
32	F	63	No	Psammomatous (with transitional areas)	I	Cranial base	Severe (+++)	D
33	M	58	Yes	Transitional	I	Parasagittal	Light (+)	−22
34	F	66	No	Psammomatous (with transitional areas)	I	Convexity	No	−22
35	F	76	No	Transitional	I	Convexity	Moderate (++)	−(1p/7/14/15/18/22) +1q
36	F	54	No	Angiomatous (with secretory areas)	I	Tentorial	Light (+)	D
37	M	54	No	Meningothelial	I	Convexity	No	−(1p/18/22) +9
38	M	68	No	Transitional	I	Parasagittal	Severe (+++)	+(1q/7/14/15/17) ++18
39	F	69	No	Psammomatous	I	Spinal	No	−22
40	M	48	No	Transitional	I	Parasagittal	Severe (+++)	−(1p/22)
41	F	66	No	Transitional	I	Parasagittal	No	−22
42	F	61	No	Meningothelial	I	Cranial base	No	D
43	M	30	No	Transitional	I	Parasagittal	No	D
44	M	66	No	Transitional	I	Convexity	Moderate (++)	−(1p/22/Y) +1q
45	F	53	No	Meningothelial	I	Cranial base	No	D
46	M	77	No	Meningothelial (with xanthomatous areas)	I	Cranial base	No	−22
47	F	48	No	Fibroblastic (with transitional areas)	I	Parasagittal	No	−22
48	F	51	No	Meningothelial	I	Cranial base	No	D
49	M	66	No	Meningothelial	I	Intraosseous	No	−1p
50	F	69	No	Psammomatous	I	Cranial base	Moderate (++)	D
51	F	72	No	Transitional	I	Convexity	Moderate (++)	−(1p/14/18/22) +1q

WHO, World Health Organization; D, diploid; −22, monosomy 22 or del(22q).

Table 2. Antibody Reagents Used for the Immunophenotypic Analysis of Meningiomas

Specificity	Antibody	Source	Clone
Major histocompatibility complex molecules	HLA-I-FITC	IOTest/Immunotech	B9.12.1
	HLA-DR-FITC	BD Biosciences	L234
Complement regulatory proteins	CD55-FITC	BD Pharmingen	IA10
	CD59-FITC	Immunostep SL	VJ1/12.2
Cellular adhesion molecules	CD2-FITC	BD Biosciences	S5.2
	CD22-FITC	BD Pharmingen	HIB22
	CD44-PE	Immunostep SL	HP2/9
	CD56-PE	Cytognos SL	C5.9
	CD58-PE	BD Biosciences	L306.4
	CD99-PE	BD Pharmingen	TÜ12
Transmembrane proteins of the tetraspanin family	CD9-FITC	IOTest/Immunotech	ALB6
	CD37-FITC	BD Pharmingen	M-B371
	CD53-PE	BD Pharmingen	HI29
	CD63-FITC	IOTest/Immunotech	CLBGran/12
	CD81-PE	BD Pharmingen	JS-81
Ectoenzymes	CD13-PE	BD Biosciences	L138
	CD38-FITC	Cytognos SL	LD38
Antiapoptotic protein	Bcl2-FITC	Dako	124
Growth factor receptors	EGFR-PE	BD Pharmingen	EGFR.1
	HER2/neu-PE	BD Biosciences	Neu 24.7
	IGFR-PE	BD Pharmingen	1H7
Other molecules	CD3-PacB	BD Pharmingen	UCHT1
	CD4-PacB	BD Pharmingen	RPA-T4
	CD8-FITC	BD Biosciences	SK1
	CD14-PE	BD Biosciences	MφP9
	CD16-FITC	IOTest/Immunotech	3G8
	CD19-FITC	BD Biosciences	4G7
	CD25-PE	BD Biosciences	2A3
	CD28-PE	BD Biosciences	L293
	CD33-PE	BD Biosciences	P67.6
	CD45-PacB	Dako	T29/33
	CD45-PacO	Invitrogen	HI30
	CD68-FITC	An der Grug	Ki-M7
	CD69-PE	BD Biosciences	L78
	CD127-FITC	BD Pharmingen	HIL-7R-M21
	CD200-PE	BD Pharmingen	MRC OX-104

ences), CD19-FITC (BD Biosciences), and CD56-PE (Cytognos SL). In a subset of samples, regulatory T cells (Tregs) and costimulatory molecules were analyzed using CD4-PacB (BD Pharmigen), CD25-PE (BD Biosciences), CD127-FITC (BD Pharmigen), and CD28-PE (BD Biosciences). $CD4^{+}CD25^{hi}CD127^{-/lo}$ Tregs were detectable at a frequency of 1 Treg in 30,000 acquired cellular events. Staining for cytoplasmic markers (bcl2 and CD68) was performed after incubation of cells (1 hour for freshly frozen tissues and overnight for fresh tissues, at −20°C) in a citrate buffer [250 mmol/L sucrose (Sigma-Aldrich), trisodium citrate 40 mmol/L (Sigma-Aldrich), and dimethyl sulfoxide 5% v/v (Merck) (pH 7.6)] as described elsewhere.[19] Staining for DRAQ5 was performed 5 minutes before the measurement in the flow cytometer.[20] As a negative control, an aliquot of each tumor sample stained only with DRAQ5 was measured in parallel to evaluate the autofluorescence levels of the distinct cell populations contained in it.

Data acquisition was performed for $\geq 1 \times 10^5$ cells per antibody combination in a FACSCanto II flow cytometer (BD Biosciences), using the FACSDiva software version 6.0 (BD Biosciences). The INFINICYT software (Cytognos SL) was used for data analysis devoted to the evaluation of the percentage of positive cells and of the amount of protein expression per cell (mean fluorescence intensity) for each marker within a cell population. An antigen was considered positive when the percentage of positive cells was >20% or the mean fluorescence intensity exceeded the mean fluorescence intensity ± 3 SDs of the baseline autofluorescence levels of unstained cells.

Fluorescence-Activated Cell Sorting and Morphologic/Genetic Characterization of the Sorted Cell Populations

Purification of different cell populations coexisting in meningioma samples was performed in 12 freshly obtained tumor samples using a 4-way fluorescence-activated cell sorter (FACSAria; BD Biosciences) and the FACSDiva software (BD Biosciences). Before sorting, cells were stained with CD45-PacB/HLA-DR-FITC/CD44-PE/DRAQ5, as described. Four different nucleated cell populations ($DRAQ5^{hi}$) were isolated (purity, >90%; mean ± SD, 96%±3%, 94%±4%, 98%±1%, and 97%±2%, respectively) based on the following phenotypes: i) sideward light scatter $(SSC)^{lo}CD45^{hi}CD44^{hi}$; ii) $SSC^{lo}CD45^{+}HLA\text{-}DR^{hi}CD44^{-}$; iii) $SSC^{lo}CD45^{-}HLA\text{-}DR^{-}CD44^{-}$, and iv) $SSC^{hi}CD45^{-}HLA\text{-}DR^{+}CD44^{+}$.

The four sorted cell populations were placed in both methanol/acetic acid 3:1 (v/v) for further interphase fluorescence *in situ* hybridization (iFISH) analysis with the 9p34/22q11.2 dual color probe (Vysis Inc., Downers Grove, IL)[21] and the PreservCyt solution (Cytyc Corporation, Boxborough, MA) for further morphologic studies. For the later studies, slides were prepared using the ThinPrep 5000 (Cytyc Corporation) automated slide processor, stained with the Papanicolaou stain using the Shandon Varistain Gemini automated instrument (Thermo Fisher Scientific Inc., Waltham, MA) and analyzed in an Olympus BX5 microscope equipped with a 100× oil objective (Olympus, Melville, NY).

Phagocytic and Endocytic Studies

The phagocytic activity of the different cell populations present in the tumor ($n = 5$) was evaluated through their ability to uptake FITC-conjugated *Esherichia coli*, using the PHAGOTEST reagent kit (Orpegen Pharma, Heidelberg, Germany).[22] In parallel, the endocytic capacity of the same cell populations was investigated in another group of tumors ($n = 7$) through the ability of cells to capture antigens at 37°C versus 4°C (control), using a conventional dextran-FITC (Sigma-Aldrich) uptake assay.[23] For both phagocytic and endocytic assays, samples were counterstained with CD45-PacO, HLA-DR-PacB, CD44-PE, and DRAQ5 to allow identification of the different cell subpopulations present in the sample.

Gene Expression Profiling Studies

In a subset of 13 tumors, the GEP was analyzed (Human Genome 133A Affymetrix array; Affymetrix Inc., Santa Clara, CA). After thawing, tumors were homogenized (Potter-'S'-Elvehjem homogenizer; Uniform, Jencons, UK), and the total RNA was isolated in two steps using TRIzol (Invitrogen) and the RNeasy Mini Kit (Qiagen, Valencia, CA). The integrity and purity of the extracted RNA were determined using a microfluidic electrophoretic system (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA). The GEPs were analyzed according to the manufacturer's instructions, using the one-cycle cDNA synthesis kit and the Poly-A RNA gene chip control kit (Affymetrix Inc.) as reported elsewhere.[24] Data files containing data about the expression levels for the 13 tumors were normalized with robust microarray normalization and analyzed using the R (*http://www.r-project.org*) and Bioconductor (*http://www.bioconductor.org*) software tools.

Statistical Analysis

Comparisons between groups were performed by the *U*-test or Wilcoxon test (for continuous variables), and Spearman's correlation or a lineal regression model were used to explore the degree of correlation among different variables (SPSS statistical software version 12.0; SPSS Inc., Chicago, IL).

Results

Immunophenotypic Identification and Characterization of Meningioma Cell Populations

Immunophenotypic analysis of meningioma samples ($n = 51$) systematically showed the presence of multiple cell populations, which included both reactive/inflammatory and neoplastic cells (Figure 1). Expression of CD45 was restricted to approximately one-fourth of the cells (24%±20%), whereas most cells in the tumor samples (76%±20%) corresponded to $CD45^-$ neoplastic cells (Figure 1B). Infiltrating $CD45^+$ inflammatory cells included two distinct populations (Figure 1C). The first one showed a SSC^{lo} and $CD45^{hi}$ phenotype (Figure 1, C–E), compatible with that of $CD3^+$ T cells (1.4%±1.5%), mostly $CD8^+$ (1.1%±1.3% of the cells, Figure 1I), and $CD3^-/CD19^-/56^+$ natural killer (NK) cells (0.2%±0.3% of the cells, Figure 1J). Of note, $CD4^+CD25^{hi}CD127^{-/lo}$ Tregs were only found in 4 of 12 cases analyzed, where they represented 5%±4% of all $CD4^+$ T cells. Expression of the CD28 co-stimulatory molecule was detected in 32%±23% and 62%±24% of the $CD8^+$ and $CD8^-$ T cells, respectively. B cells were detected in 58% of the tumors at very low frequencies (0.03%±0.05% of the cells). The second population of $CD45^+$ cells revealed surface membrane reactivity for HLA-DR^+, $CD14^+$, and $CyCD68^+$ (22%±18% of the overall cellularity) and variable positivity for CD16 (47%±20%) and CD33 (39%±32%), an immunophenotype consistent with that of tissue macrophages (Figure 1, C, F, and H). These latter $CD45^+$HLA-$DR^+CD14^+CD16^{-/+}CD33^{-/+}$ cells systematically expressed the CD9, CD53, CD63, and CD81 tetraspanin molecules, the CD55 and CD59 complement regulatory proteins, and HLA-I. The CD38 ectoenzyme, the CD2 and CD44 cellular adhesion proteins, and the bcl-2 anti-apoptotic protein were also detected in all cases, although in variable percentages of these cells (Table 3). In turn, expression of CD13, CD99, CD58, CD22, CD69, and CD37 was detected in these $CD45^+$HLA-DR^+CD14^+ cells from only a subgroup of tumors (Table 3). The EGFR, IGFR, and HER2/neu growth factor receptors and the CD200 protein were constantly negative in this cell population. In addition, these cells had significant phagocytic ($P = 0.009$) and endocytic ($P = 0.002$) activity at 37°C (Figure 2). Interestingly, no significant differences were found regarding the distribution of inflammatory cells and their subsets according to the degree of brain edema and the distinct histologic subtypes (data not shown).

$CD45^-$ tumor cells displayed variable light scatter, HLA-DR, and CD44 fluorescence levels, and they consisted of two clearly defined subsets: $SSC^{lo}CD45^-$HLA-DR^-CD44^- (23%±23%), and $SSC^{hi}CD45^-$HLA-DR^+CD44^+ (53%±24%) events. The latter cell population systematically displayed high reactivity in all cells for the CD9, CD63, and CD81 tetraspanin molecules, the CD55/CD59 complement regulatory proteins, HLA-I, and the CD13 ectoenzyme. Other proteins that were expressed by this cell population in

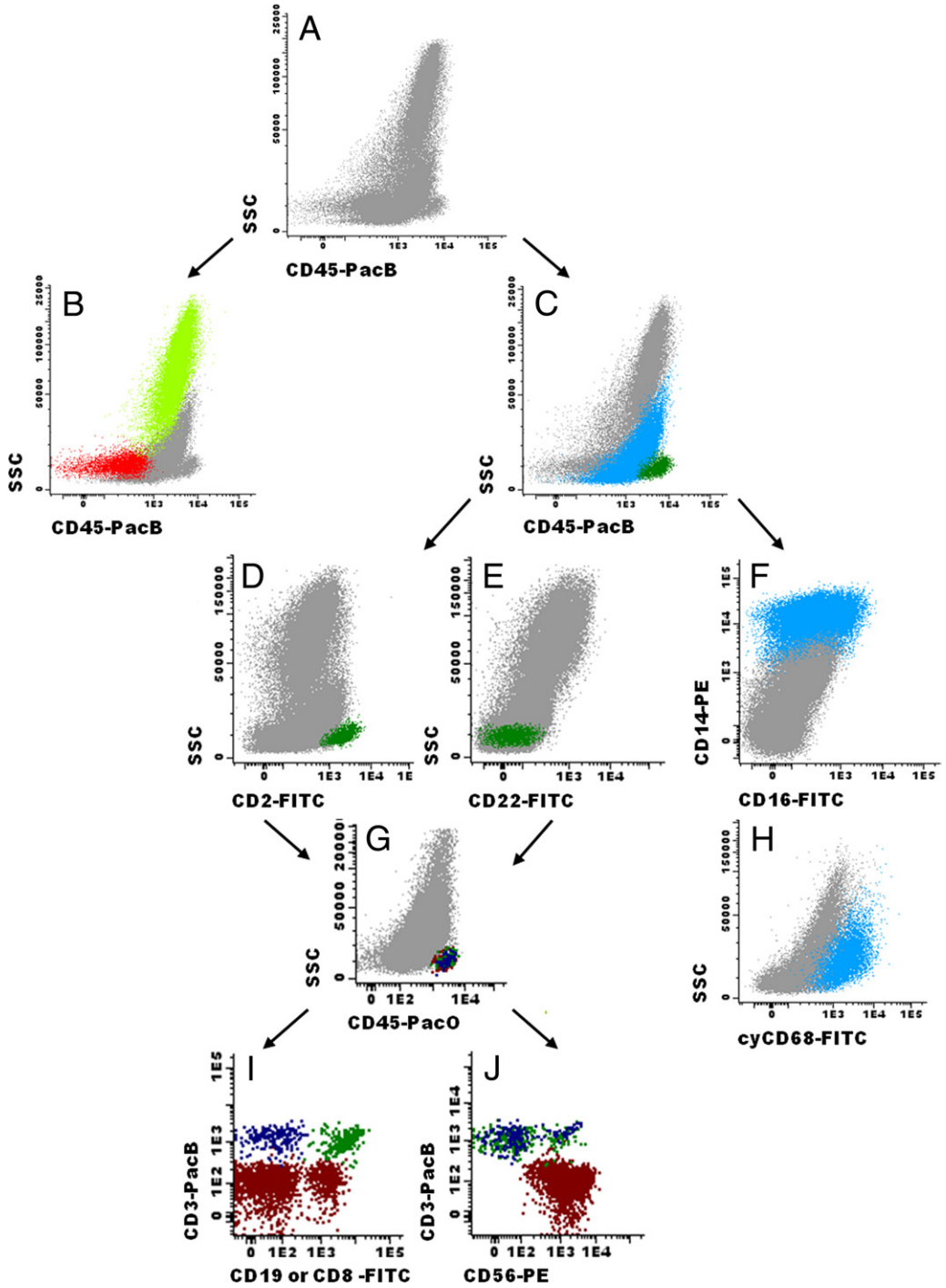

Figure 1. Multiparameter flow cytometry immunophenotypic identification of different cell compartments in meningioma tissue samples. **A:** Overall SSC versus CD45 pattern of reactivity for all nucleated cell compartments ($DRAQ5^+$) in a representative single cell suspension from a meningioma tissue specimen. Meningeal tumor cells (red and green dots) (**B**) and $CD45^+$ inflammatory cells (**C**) infiltrating the tumor (blue and dark green dots); these latter infiltrating $CD45^+$ cells corresponded to lymphocytes expressing CD2 in the absence of CD22 (dark green dots in **D** and **E**), composed of $CD8^+$ (dark green dots in **G**, **I**, and **J**) and $CD8^-$ (dark blue dots in **G**, **I**, and **J**) T lymphocytes plus NK cells (dark red dots in **G**, **I**, and **J**), and $CD45^+HLA\text{-}DR^{hi}CD44^{het}$ antigen-presenting cells (blue dots in **C**, **F**, and **H**) showing a $CD14^+CD68^+CD16^{-/+}$ immunophenotype.

most meningiomas (partial expression) were IGFR, HER2/neu, EGFR, CD14, CD38, and bcl-2 (Table 3). In addition, other proteins, such as CD53, CD58, CD200, CD99, and CD2, were only present in a subset of cells from a lower percentage of cases, whereas CD16, CD22, CD37, and CD69 were systematically negative (Table 3). $SSC^{hi}CD45^-HLA\text{-}DR^+CD44^+$ tumor cells showed no detectable phagocytic activity, whereas they displayed an endocytic activity similar to that of tissue macrophages (Figure 2). In addition, a significant correlation was found between the percentage of $SSC^{lo}CD45^+HLA\text{-}DR^{hi}CD44^{het}$ inflammatory cells and both the amount of expression of

Table 3. Patterns of Protein Expression of Neoplastic Cells and Antigen-Presenting (Inflammatory) Cells Infiltrating the Tumor in Meningiomas ($n = 51$)

	Meningeal-tumor cells ($SSC^{hi}/CD45^{-}/HLA\text{-}DR^{+}/CD44^{+}$ cells)		Antigen-presenting inflammatory cells ($CD45^{+}/HLA\text{-}DR^{hi}/CD14^{+}/CD16^{-/+}$)	
Protein	No. of positive samples/total samples (%)	Positive cells, %*	No. of positive samples/total samples (%)	Positive cells, %*
CD9	51/51 (100)	97 ± 10	18/18 (100)	99 ± 3
CD44	51/51 (100)	80 ± 26	18/18 (100)	66 ± 18
CD55	51/51 (100)	96 ± 12	18/18 (100)	98 ± 4
CD59	51/51 (100)	99 ± 2	18/18 (100)	100 ± 1
CD63	51/51 (100)	90 ± 15	18/18 (100)	87 ± 11
CD81	51/51 (100)	98 ± 5	18/18 (100)	98 ± 3
HLA-I	51/51 (100)	90 ± 14	18/18 (100)	100 ± 0
CD13	51/51 (100)	89 ± 15	15/18 (83)	63 ± 24
HER2/neu	49/51 (96)	73 ± 26	0/18 (0)	
IGFR	49/51 (96)	73 ± 25	0/18 (0)	
EGFR	40/51 (78)	69 ± 24	0/18 (0)	
CD200	30/51 (59)	46 ± 22	0/18 (0)	
CD38	44/51 (86)	66 ± 27	18/18 (100)	81 ± 22
Bcl2	44/51 (86)	65 ± 24	18/18 (100)	64 ± 26
HLA-DR	41/51 (80)	69 ± 23	18/18 (100)	98 ± 3
CD14	39/51 (76)	76 ± 19	18/18 (100)	99 ± 1
CD53	32/51 (63)	62 ± 24	18/18 (100)	95 ± 8
CD2	11/51 (22)	57 ± 24	18/18 (100)	79 ± 25
CD58	30/51 (59)	61 ± 22	13/18 (72)	81 ± 15
CD99	24/51 (47)	60 ± 21	14/18 (78)	53 ± 20
CD45	0/51 (0)		18/18 (100)	92 ± 7
CD16	0/51 (0)		13/18 (72)	47 ± 20
CD22	0/51 (0)		8/18 (44)	51 ± 19
CD69	0/51 (0)		7/18 (39)	24 ± 3
CD37	0/51 (0)		5/18 (28)	30 ± 5

*Results expressed as mean ± SD.

HLA-DR ($r^2 = 0.4$, $P = 0.001$) and CD14 ($r^2 = 0.4$, $P = 0.001$) and the percentage of neoplastic cells that were positive for these two markers ($r^2 = 0.3$, $P = 0.02$, and $r^2 = 0.4$, $P = 0.005$, respectively). As discussed in the following section, $SSC^{lo}CD45^{-}HLA\text{-}DR^{-}CD44^{-}$ tumor cells had absence of expression of all markers evaluated.

Noteworthy, among $SSC^{hi}CD45^{-}HLA\text{-}DR^{+}CD44^{+}$ cells a significant correlation ($r^2 \geq 0.5$; $P \leq 0.02$) was found between the mean amount of expression of each protein/cell in paired fresh and freshly frozen tissue samples ($n = 18$), except for Cybcl2, CD2, and CD200 (Table 4). Despite such correlation, significantly higher levels were observed for freshly frozen cells for CD2, CD14, CD53, CD55, CD63, CD99, and HLA-DR ($P < 0.05$; Table 4).

Morphologic and Genetic Features of Purified Cell Populations

Morphologic and genetic analyses performed on highly purified cell populations confirmed coexistence of meningeal tumor cells and nonmeningeal infiltrating inflammatory cells in every meningioma sample (Figure 3). Accordingly, both $SSC^{lo}CD45^{-}HLA\text{-}DR^{-}CD44^{-}$ and $SSC^{hi}CD45^{-}HLA\text{-}DR^{+}CD44^{+}$ cells displayed the cytogenetic alterations detected in the tumor, eg, del(22q), in association with morphologic features consistent with those of tumor cells (eg, large nuclei, granular chromatin, and thick nuclear membrane); whereas $SSC^{hi}CD45^{-}HLA\text{-}DR^{+}CD44^{+}$ cells had abundant cytoplasm, $SSC^{lo}CD45^{-}HLA\text{-}DR^{-}CD44^{-}$ cells corresponded to bare nuclei (Figure 3A). In turn, the two $CD45^{+}$ cell populations systematically lacked on such genetic alterations (Figure 3, B and C). Morphologically, $SSC^{lo}CD45^{hi}HLA\text{-}DR^{het}CD44^{hi}$ cells displayed a typical appearance of mature lymphocytes, composed of round small cells with scarce cytoplasm,

Figure 2. Phagocytic and endocytic ability of neoplastic tumor cells and different subpopulations of tumor-infiltrating inflammatory cells. The phagocytic and endocytic activity (expressed as the percentage of positive cells determined by the uptake of *E coli*–FITC and dextran-FITC, respectively) of lymphocytes (black bars; negative control), tissue macrophages (gray bars), and tumor cells (white bars) is compared. *$P = 0.009$, **$P = 0.002$.

Table 4. Correlation between the Amount of Expression (MFI) of Different Proteins in Tumor Cells from Paired Fresh and Freshly Frozen Meningioma Tissue Samples ($n = 18$)

Marker	Cell localization	Mean amount of protein (MFI) per cell			Correlation coefficient	
		Fresh tissue*	Frozen tissue*	*P* value	r^2	*P* value
CD14	Membrane	481 ± 406	655 ± 542	0.03	0.9	<0.001
CD99	Membrane	186 ± 146	258 ± 247	0.03	0.9	<0.001
CD58	Membrane	258 ± 217	354 ± 334	NS	0.9	<0.001
CD13	Membrane	8771 ± 8733	9539 ± 10488	NS	0.8	<0.001
HLA-I	Membrane	8040 ± 6294	7715 ± 5575	NS	0.8	<0.001
CD9	Membrane	24,043 ± 20,352	27,243 ± 22,448	NS	0.8	<0.001
HLA-DR	Membrane	662 ± 658	1482 ± 1564	0.001	0.7	<0.001
EGFR	Membrane	473 ± 571	388 ± 367	NS	0.7	0.001
CD53	Membrane	132 ± 94	209 ± 195	0.03	0.7	0.002
IGFR	Membrane	1001 ± 905	1169 ± 984	NS	0.7	0.002
CD55	Membrane	3698 ± 1380	4686 ± 1966	0.01	0.7	0.002
CD81	Membrane	10,791 ± 12,282	9413 ± 8913	NS	0.7	0.002
HER2/neu	Membrane	1096 ± 825	1297 ± 1177	NS	0.6	0.009
CD59	Membrane	25,045 ± 19,142	28,607 ± 17,346	NS	0.6	0.009
CD38	Membrane	994 ± 1076	932 ± 1055	NS	0.6	0.01
CD44	Membrane	1425 ± 1083	1713 ± 1422	NS	0.6	0.01
CD63	Membrane	1835 ± 1011	2783 ± 1062	0.003	0.5	0.02
CD200	Membrane	550 ± 546	378 ± 348	NS	0.5	
Bcl2	Cytoplasmic	553 ± 353	684 ± 414	NS	0.4	
CD2	Membrane	173 ± 87	277 ± 133	0.005	0.2	

Markers that were systematically negative are not listed in the table.
*Results expressed as mean ± 1 SD.
MFI, mean fluorescence intensity.

whereas $SSC^{lo}CD45^{+}HLA\text{-}DR^{hi}CD44^{het}$ cells displayed an irregular nuclei and more abundant cytoplasm, with a morphologic appearance compatible with tissue macrophages (Figure 3, B and C).

Of note, the overall percentage of $CD45^{-}$ tumor cells (71%±22%) detected by MFC immunophenotyping revealed a significant correlation ($r^2 = 0.62$; $P < 0.001$) with the percentage of cytogenetically altered tumor cells detected by iFISH (65%±21%) in the same samples.

Relationship between Overall mRNA and PEPs

When considering the overall cellularity of tumor samples, a significant direct correlation was found between the mRNA and the protein expression levels for the CD13 ectoenzyme ($r^2 = 0.9$; $P < 0.001$), the CD58 ($r^2 = 0.7$; $P = 0.004$) and CD99 ($r^2 = 0.8$; $P = 0.003$) cell adhesion molecules, and HLA-DR ($r^2 = 0.7$; $P = 0.01$). Conversely, an inverse correlation ($r^2 = -0.7$; $P = 0.006$) between the mRNA and the protein levels was observed for the HER2/neu growth factor receptor (Table 5). No significant correlation was found between mRNA and protein levels for the other 17 proteins analyzed. Noteworthy, a similar pattern and degree of correlation were observed for the studied proteins, when we considered the protein expression levels specifically found for meningeal tumor cells (Table 5). Despite this, a significant direct correlation was found between the mRNA levels of the EGFR and HER2/neu growth factor receptors and the percentage of tumor cells in the sample ($r^2 > 0.5$; $P < 0.05$; data not shown), whereas an inverse correlation was found for HLA-DR, HLA-I, bcl2, CD45, CD14, CD16, CD53, and CD99 ($r^2 \leq -0.5$; $P < 0.05$; data not shown).

Discussion

Meningiomas are heterogeneous tumors that consist of both neoplastic cells and other infiltrating nonimmune and immune cells (eg, macrophages/microglial cells and lymphocytes); the latter cells have been suggested to play an important role in modulating the growth and immunogenicity of meningiomas.[3–5,7] Although each of these cellular components displays a uniquely different gene expression mRNA and protein profile, to the best of our knowledge, no study has been reported so far in which the most represented cell populations have been systematically identified and characterized in meningioma samples.

Overall, our results confirm the heterogeneous cellular composition of meningiomas, which, together with a major fraction of neoplastic cells, systematically reveals variable infiltration by tissue macrophages and, to a lesser extent, T, NK, and a few B cells. Simultaneous identification of the different cell populations was optimally achieved based on differential MFC patterns of expression of CD45, HLA-DR, and CD44 by nucleated cells ($DRAQ5^{hi}$). Based on CD45, two major groups of cells were identified: $CD45^{-}$ neoplastic cells and $CD45^{+}$ infiltrating immune cells. Among the latter cells, most have an $HLA\text{-}DR^{+}CD14^{+}CD68^{+}CD16^{-/+}CD33^{-/+}$ phenotype consistent with a monocytic/macrophage lineage origin, as also supported by their high phagocytic and endocytic ability and their morphologic appearance. Together, these findings support previous observations that reported infiltration by macrophages in meningiomas.[3–5,7] However, although multiple reports describe infiltration of different tumor types by tissue macrophages,[25] little is

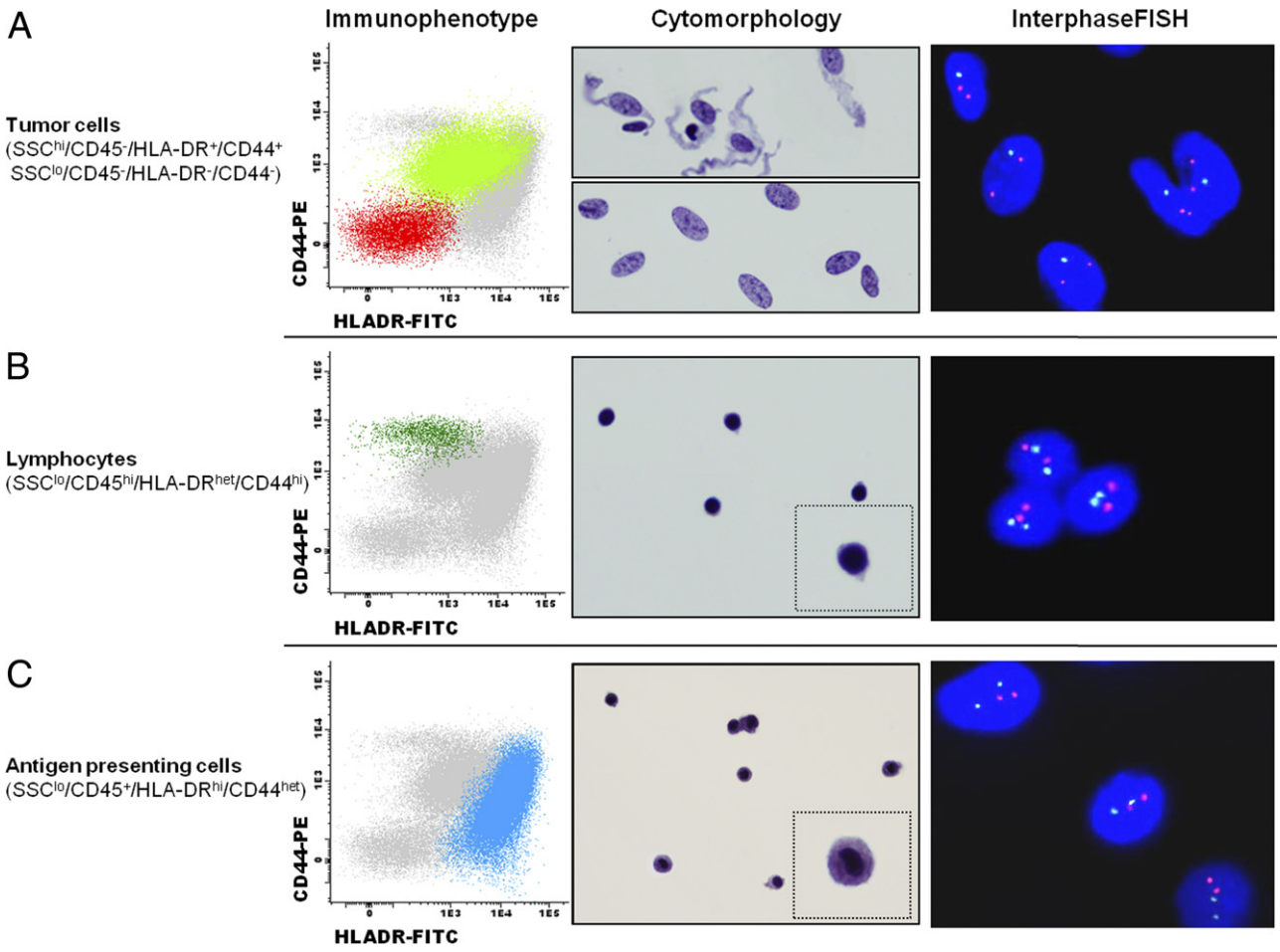

Figure 3. Cytomorphologic and genetic characterization of fluorescence-activated cell sorted cell populations present in a representative meningioma tissue sample. On the basis of multiparameter flow cytometry immunophenotyping, three major populations of cells were systematically identified: tumor cells (**A**), lymphocytes (**B**), and antigen-presenting cells (APC) (**C**). Among the tumor cell population, two subsets were also found (FSC/SSC^{hi}CD45^{-}HLA-DR^{+}CD44^{+} light green dots and FSC/SSC^{lo}CD45^{-}HLA-DR^{-}CD44^{-} red dots; **A**, **left**). Lymphocytes systematically revealed a FSC/SSC^{lo}CD45^{hi}HLA-DR^{het}CD44^{hi} phenotype (dark green dots; **B**, **left**), whereas tissue macrophages presented a FSC/SSC^{lo}CD45^{+}HLA-DR^{hi}CD44^{het} phenotype (blue dots; **C**, **left**). Cytomorphologic features of the two subsets of tumor cells, lymphocytes, and tissue macrophages as per the Papanicolaou stain (original magnification, ×1000) are shown in the **middle panels** of **A**, **B**, and **C**, respectively; the images on the **right panels** show iFISH analysis of chromosomes 9p34 and 22q11.2 (9p34/22q11.2 red/green dual color probe), with two copies of chromosomes 9 and 22 in all lymphocytes and tissue macrophages, whereas tumor cells from the same meningioma sample displayed del(22q), as reflected by a single green spot/nuclei. No differences were observed between the iFISH probes in the two subsets of tumor cells, but cytomorphologic analysis revealed a disrupted cytoplasm with bare nuclei in one of them (**A**, **middle**).

known about their phenotype and functional properties in meningiomas. As could be expected, tumor macrophages expressed HLA-I and the CD55 and CD59 complement regulatory proteins, in association with partial positivity for the CD13 (aminopeptidase N)[26] and CD38 ectoenzymes,[27] bcl2,[28] activation-induced CD69,[29] multiple adhesion molecules (eg, CD2,[30] CD44,[31] CD58,[32] and CD99[33]), and several tetraspanins, involved in the organization of microdomains essential for the regulation of signaling pathways central to macrophage activation.[34] These infiltrating immune cells may play an important role in tumor immunology through complex relationships with tumor cells and other cells in the tumor microenvironment.[25] Currently, tissue macrophages are grouped into M1 and M2 cells, according to the pattern of cytokines they secrete.[25] Several studies suggest that tumor infiltrating macrophages (eg, in gliomas) exhibit features of M2-like macrophages,[35–37] promoting tumor progression.[38] However, M1-like macrophages have also been detected in some tumors where they are associated with a better prognosis.[39,40] Further studies are required to determine the M1/M2-like nature of tissue macrophages in meningiomas and their impact on the disease.

Other less represented CD45^{+} immune cells (CD45^{hi}) corresponded to cytotoxic T CD8^{+} and NK cells. These results confirm previous findings which show that such tumor-infiltrating lymphocytes in meningioma[3] and also other tumors[41] mainly consist of CD8^{+} cytotoxic T nevertheless, these cells are frequently unable to control tumor growth and progression.[41] Whether this is due to a specific functional defect of such cytotoxic cells, associated or not with an inhibitory effect induced by standing Tregs, remains to be elucidated. In this regard, our results reveal the absence of CD4^{+}CD25^{hi}CD127^{lo} Tregs in most of the meningiomas analyzed, supporting the lack of local immune tolerance induced by Tregs. Recent studies indicate that the presence of Tregs in various cancer types correlates with a poor prognosis.[42] In line

Table 5. Correlation between the mRNA Levels and the Mean Amount of Protein Expressed per Cell for 22 Markers Analyzed in 13 Meningiomas (Protein Levels Were Evaluated for the Overall Cellularity of the Sample and Specifically for the Meningioma Tumor Cells)

MFC protein	Correlation coefficient			
	Overall cellularity		Meningeal tumor cells	
	r^2	*P* value	r^2	*P* value
CD45	0.5		0.2	
HLA-I	0.01		−0.07	
CD81	0.1		0.3	
CD9	−0.3		−0.1	
CD13	0.9	0.0001	0.8	0.001
CD38	0.04		−0.1	
EGFR	−0.05		0.2	
CD2	0.2		−0.2	
CD99	0.8	0.003	0.7	0.009
CD16	0.1		−0.5	
CD14	0.4		0.3	
HLA-DR	0.7	0.01	0.7	0.01
CD44	0.4		0.4	
CD55	−0.5		−0.4	
CD53	0.5		0.5	
CD63	−0.3		−0.1	
IGFR	0.1		0.2	
HER2Neu	−0.7	0.006	−0.6	0.02
CD59	−0.1		0.2	
CD58	0.7	0.004	0.7	0.01
Bcl2	0.5		0.6	0.05
CD69	0.5		0.1	

For mRNA levels, the mean value obtained for all probes in the array specific for the corresponding protein mRNA was used. Only significant *P* values are shown.

with these observations, Jacobs et al[43] also reported the virtual absence of Tregs in meningiomas compared with other malignant tumors, such as gliomas. Furthermore, our results also show that tumor-infiltrating T cells co-express CD28, a molecule that is critical for providing co-stimulatory signals required for T-cell activation. This, together with the observed expression of antigen-presenting molecules (both HLA class I and class II) by tumor cells, points to a potential role of inflammatory infiltrates of meningiomas in controlling tumor growth.

Two distinct groups of $CD45^-$ neoplastic cells were found by MFC immunophenotyping. However, sorting of the two populations revealed that despite the fact that they both carried the same cytogenetic markers, only one corresponded to live cells with a $SSC^{hi}CD45^-HLA\text{-}DR^+CD44^+$ phenotype and typical morphologic characteristics of meningioma cells. The other $SSC^{lo}CD45^-HLA\text{-}DR^-CD44^-$ subset consisted of bare nuclei, probably generated during sample preparation by the mechanical disaggregation and/or the freezing procedures. This finding helps explain the absence of expression of virtually all proteins analyzed in this later population. Therefore, a question remains about whether MFC immunophenotyping provides a reliable tool to assess protein expression in frozen meningioma samples. In this regard, it should be emphasized that fresh tumor samples are frequently not readily available for routine MFC immunophenotyping. Therefore, a major goal of our study was to determine the impact of freezing on the pattern of protein expression by meningioma cells. Overall, our results revealed a significant correlation for most proteins analyzed in fresh versus freshly frozen tumor samples; despite this, higher protein levels were frequently found after freezing. Such differences could be due to a better preservation of the PEPs in frozen samples; however, they may also reflect simultaneous detection of proteins at the membrane and cytoplasmic cell compartments due to permeabilization of cells induced by the freezing process. Further studies are required to confirm these hypotheses; meanwhile, our results support use of frozen instead of fresh tumor tissues for more accurate MFC evaluation of the overall PEP in meningiomas.

Another major goal of our study was to determine the relationship between GEPs at the mRNA versus the protein level. Interestingly, CD13, CD58, CD99, and HLA-DR were the only proteins for which a significant correlation between protein and mRNA levels was observed. For the other proteins analyzed, either no correlation or an even negative significant correlation (eg, HER2/neu) was found. Altogether, these results indicate that mRNA levels frequently do not reflect the amount of protein expressed by individual tumor cells.[44] Such discrepancies could be expected because the level of expression of membrane proteins depends not only on their synthesis but also on other factors, such as posttranslational modifications; a balance among protein synthesis, degradation, and secretion; and/or the mobilization of previously stored proteins, as extensively described in previous studies comparing mRNA and protein levels.[44–46] Of note, the integrity of the extracted RNA determined by microfluidic electrophoresis was confirmed in our study by the high-quality RNA obtained, which would rule out potential RNA degradation during tumor disaggregation procedures. Similarly, mechanical disaggregation of the tissue required for MFC immunophenotyping could also have an impact on the levels of expression of proteins in individual cells. If this holds true, then the negative correlation observed between protein and mRNA levels for some markers, ie, between HER2/neu mRNA and protein levels, could not be clearly explained. In fact, such inverse correlation potentially reflects specific internalization and/or cleavage of HER2/neu due to recycling of the receptor between the plasma membrane and the endosomal compartments, in addition to protease-mediated cell surface cleavage in activated cells.[47] In line with this hypothesis, previously reported studies indicate that mechanical disaggregation is a better technique than enzymatic methods for protein evaluation in individual cells from solid tumor samples by MFC immunophenotyping.[48,49] Because mRNA studies are performed with the whole tumor sample, including both neoplastic and infiltrating immune cells, we wondered whether the cellular heterogeneity of the tumor could also have an impact on the GEPs. Interestingly, a positive correlation was found between the mRNA levels of proteins specifically expressed by tumor cells (eg, EGFR and HER2/neu) and the percentage of neoplastic cells in the tumor sample, whereas markers highly (or exclusively) expressed by the infiltrating inflammatory cells (eg, HLA-DR, HLA-I, bcl2, CD45, CD14,

CD16, and CD53) were inversely correlated with the tumor cell contents of the sample. Together, these results indicate that microarray-based mRNA expression profiles partially reflect the cellular composition of the tumor rather than precise features of cancer cells, whereas evaluation of gene expression at the protein level by MFC immunophenotyping would more closely reflect the phenotypic profile of neoplastic cells.

Detailed MFC analysis of the immunophenotypic characteristics of tumor cells in meningiomas revealed constant expression of several adhesion-associated molecules, such as the CD13 ectoenzyme,[50] CD44,[51] and CD9, CD63, and CD81 tetraspanins,[52–55] which may play an important role in the regulation of tumor cell motility, proliferation, and intracellular signaling. Interestingly, CD13 expression in meningiomas has been previously reported to be inversely associated with a more indolent disease behavior,[56] in line with the high levels of CD13 detected in our cohort, mainly composed of World Health Organization grade I meningiomas. In addition, expression of CD44, which has been also previously described in meningiomas,[15,57] emerges as a potentially relevant molecule in these tumors because signaling through CD44 inhibits merlin, a protein coded in chromosome 22 whose expression is frequently lost in meningiomas.[58,59] Conversely, to the best of our knowledge, this is the first study in which expression of tetraspanins is analyzed in meningioma cells, showing a unique pattern of CD9, CD63, and CD81 expression, associated with variable levels of CD53, in the absence of CD37.

Previous studies have highlighted the relevance of antiapoptotic proteins[60] and growth factor receptors[61–65] in meningioma cell growth and survival due to their association with both tumor histopathologic findings and patient outcome.[61,62,65–67] In line with these observations, we found heterogeneous patterns of expression of HER2/neu, IGFR, and EGFR in meningiomas, together with variable levels of positivity for the antiapoptotic bcl-2 protein. Further studies, in which the impact of the patterns of expression of these proteins is investigated, are required to determine their clinical value.

In recent years, tumor cell lysis through complement-activated proteins has been identified as a relevant cytotoxic mechanism that could be exploited for novel cancer-targeted therapies. Interestingly, Shinoura et al[68] reported low mRNA expression of the CD55 and CD59 complement regulatory molecules in meningiomas, which would support targeting tumor cells by such therapies. However, our results reveal high levels of both proteins on the tumor cell membrane, which would potentially protect them from bystander injury when complement is activated.[69] Interestingly, expression of HLA-I was also systematically detected in meningioma cells, which could favor the control of tumor growth because HLA-I is involved in the presentation of tumor self-antigens during immune responses by cytotoxic cells against intracellular proteins.[70]

Noteworthy, meningioma cells from most tumors shared expression of two molecules characteristic of tissue macrophages: HLA-DR and CD14. Despite the fact that HLA class II antigen expression is generally restricted to professional antigen-presenting cells and thymic epithelial cells, HLA class II$^+$ tumor cells have also been recurrently found in breast and colorectal carcinomas, in association with a better patient outcome.[71,72] This finding could be related to the fact that HLA class II$^+$ tumor cells may facilitate induction of antitumor T-cell responses by CD4$^+$ T_H1 lymphocytes, indicating that expression of determinants of the immune response by tumor cells may influence tumor progression and patient outcome.[71,73] Interestingly, in the present study we demonstrate that tumor cells not only co-express HLA-II (eg, HLA-DR) but also display a significant endocytic activity, a function typically required by distinct cell types, including antigen-presenting cells, to up-regulate expression of HLA-II.[74] This finding, together with the expression of the Toll-like receptor–associated CD14 molecule, suggests that neoplastic cells from meningiomas could play a critical role in priming and controlling local inflammatory and T-cell immune responses. In contrast to glial cells, meningeal cells have not been ontogenetically linked with the monocyte, macrophage, and dendritic cell lineages. However, expression of HLA-DR by meningioma cells has been previously reported by others.[2,3] Likewise, expression of CD14 has also been found in cell types other than the monocytic/macrophage lineage,[75–78] including meningeal cells.[79,80] On the basis of similar observations, Shabo et al[81] suggested that such mixed phenotypes could result from heterotypic cell fusion between primary cancer cells and tumor-associated macrophages. However, the absence of DNA aneuploidy in most meningioma cells, as assessed by the DRAQ5 and other DNA staining,[82] would rule out such a possibility. Further studies are necessary to better understand the role of HLA class II$^+$ tumor cells in meningiomas.

In summary, we propose a new four-color MFC-based strategy for the evaluation of the cellular composition of meningiomas. Overall, our results reveal a systematic presence of inflammatory and other immune cells coexisting with variable numbers of neoplastic cells, such infiltrating inflammatory cells mainly consisting of tissue macrophages and, to a lesser extent, cytotoxic T CD8$^+$ and NK cells. Further analysis of the PEPs in fresh versus frozen samples revealed identical profiles, although the freezing process may have a moderate impact on preserving the levels of expression of individual proteins. At last, here we report that MFC immunophenotyping provides a more reliable way of assessing gene expression by tumor cells at the protein/functional level compared with mRNA levels assessed by microarrays.

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