MOLECULAR MARKERS OF BRAIN TUMOR CELLS

Molecular Markers of Brain Tumor Cells

Implications for Diagnosis, Prognosis and Anti-Neoplastic Biological Therapy

by

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PREFACE

The last twenty years of brain tumor research has seen immunohistochemistry applied and develop from an experimental research technique to a nearly routine method of great importance in histopathology. The field of morphologic research in oncology has been revitalized and revolutionized by immunohistochemistry in that now functional aspects can be easily associated with morphological descriptions. It comes as no surprise that the scientific conferences of the past decade have generated great interest since immunohistochemistry has allowed researchers to development epoch-making discoveries in molecular oncology, practically delving into the molecular biologic aspects of cancerogenesis, cellular neoplastic transformation and the intimate mechanisms of neoplastic progression, and metastasis along with significant expansion of our knowledge concerning the processes that govern cell cycle, cell proliferation and differentiation, apoptosis, immune surveillance, angiogenesis and signal transduction control, without sacrificing the beauty of classical morphology.

The development of immunohistochemistry to its present place in research, diagnostics and therapy, of course, could not have been possible without the discovery of the methodology of monoclonal antibody production, one of the most important scientific discoveries of the twentieth century.

GROWTH FACTORS IN MAMMALIAN EMBRYOGENESIS AND NEOPLASTIC TRANSFORMATION

The elucidation of the molecular mechanisms underlying embryonic growth control is a key step in the attempt to understand embryonic development and the regulation of cell proliferation and its impairment in neoplastic transformation (1). The most extensive cellular proliferation and differentiation takes place during early ontogenesis, therefore it seems likely that growth factors have a major role at this time both in the regulation of cell proliferation and the process of immunophenotypic (IP) differentiation. The expression of growth factors and their receptors by neoplastically transformed cells is out of control; this can lead to unchecked and continuous cell division. Malignant cells may secrete some growth factors and simultaneously express their receptors (autocrine stimulation) (2). Neoplastic cells also express the receptors for the growth factors secreted by neighboring cells (paracrine stimulation). Murray and Kirschner (3) demonstrated the primitive nature of the embryonic division cycle (lack of G1 regulation), as compared to the more complex regulation in which growth factors act to modulate the growth and differentiation of somatic cells [proline directed protein phosphorylation- (4)].

An alternative to studying the embryo *in vivo* is to use *in vitro* experimental models, such as teratocarcinoma stem cells (EC-embryonal carcinoma cells) which share numerous biochemical, morphological and immunological properties with normal early pluripotent stem cells. Some growth factors are unique, appearing only during embryonic/fetal (ontogenetic) development, others especially when derived from adult tissues can be present more permanently (defined growth factors). The data accumulated from numerous studies has defined four growth factors

involved in early embryogenesis: *1)* insulin-like growth factors (IGFs); *2)* epidermal growth factors (EGFs); *3)* transforming growth factors (TGFs) and *4)* platelet-derived growth factors (PDGFs) (5-8). PDGF also increases the production of oncogenes c-*myc* and c-*fos*. Epigenetic mechanisms appear to involve an interaction between mitogenic growth factors and factors which induce cell differentiation. Neuronal differentiation of PC12 cells was observed after microinjection of the *ras* oncogene protein (9) or after infection of these cells with *ras* containing retroviruses (10). The involvement of the *ras* protein in this process is further supported by the observation that microinjection of *ras*-specific antibodies inhibits the NGF-induced differentiation of PC12 cells (11). The mitogenic action of growth factors and the anti-proliferative effect of IFN may be independent of cell cycle events, as demonstrated in studies with vascular smooth muscle and endothelial cells. Growth factors such as TGF-β have also been implicated in allowing for the sustained growth of the neoplasm, as well as in inhibiting the anti-neoplastic immune response. It has recently been proposed that the cell membrane located receptors for peptide growth factor (PGF-R) can be regarded as specific targets for immunodetection and immunotherapy of human malignancies (12). PGF-Rs play a crucial role in the regulation of neoplastic cell proliferation and may behave as TAAs. PGF-Rs are often present in greater quantity on malignant cells and their cell surface expression is regulated by cytokines. Neoplastic cells can promote their own proliferation by secreting PGFs, which act in the paracrine and autocrine stimulation of the neoplasm mass (13, 14). PGF-R may, therefore, represent an ideal cellular target for at least two various immunotherapeutic approaches: 1) for conjugated or unconjugated MoABs and 2) for genetically engineered fusion proteins composed of PGF-R physiological ligands conjugated to genetically modified bacterial toxins. Other clinical studies have been performed describing the targeting of receptors of epidermal growth factor (EGF) and interleukin 2 (IL-2) on neoplastic cells.

The development of neoplastic cell-specific and targeted immunotherapies is of particular interest. It is great to see that clinical oncologists are now finally taking this approach, especially with all of its many unprobed possibilities. We predict that the next three decades will see the employment of individualized “cocktails” of conjugated antibody molecules, targeting multiple antigenic epitopes, as the main line of non-toxic and efficacious therapy of human neoplastic disease, especially in the treatment of residual and metastatic neoplasms.

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I. MOLECULAR BIOLOGY OF TUMORS

Chapter 1

BRAIN TUMORS

1. INTRODUCTION

In the last three decades, major advances in molecular biology and subsequently in the biology of neoplastic diseases and fundamental genetic discoveries have improved our understanding of neoplastic cellular transformation and its full blown development into an advanced neoplastic progression (1). Cancer associated markers (CAMs) represent the biochemical or immunological counterparts of the morphology of tumors. The expression of immunocytochemically defined cancer associated markers is also related to the tissue of origin and is thus, not a random event.

During the past 25 years, the employment of antigenic epitope specific MoABs against oncofetal, neoplasm associated, cell lineage specific, endothelial, and cell proliferation related antigens in the diagnosis and biological assessment of prognosis in neoplastic disease gained increased importance. A sensitive direct correlation exists between the expression of certain molecules and the development of an invasive, highly malignant IP of neoplastic cells, allowing for the occurrence of neoplasm induced neoangiogenesis and metastasis.

2. MEDULLOBLASTOMA

Primary brain tumors remain the second most common type of solid neoplasms during childhood (younger than 15 years of age) and the posterior fossa is the most common region of the central nervous system (CNS)

affected. Medulloblastomas are in fact the most common childhood brain tumor (2). The annual incidence of pediatric brain tumors appears to be on the rise caused partly by improvements of diagnostic neuroimaging and its increased availability (3). Despite significant increases in survival rate during the past decade, the great majority of pediatric patients with medulloblastomas (MEDs) or primitive neuroectodermal brain tumors (PNETs) still succumb to their disease. Advances have recently been made in the employment of chemotherapy for childhood brain tumors. Chemotherapy increases disease-free survival in high-risk MED/PNET patients and enables the reduction of radiation therapy in standard-risk patients (4). Radiation can be significantly delayed and neurotoxicity ameliorated in many infants using chemotherapy. Chemotherapy can cause reduction in size of low-grade glioma, optic glioma, and oligodendroglioma. High-grade glioma and ependymoma are relatively chemoresistant. A recent venture by scientists has been an attempt to assess the risk stratification in medulloblastomas. Gene expression profiling has been shown to predict medulloblastoma outcomes independent of clinical variables (5). In addition, Erb-B-2 expression and clinical risk factors haven been shown to together constitute a highly accurate disease risk stratification tool (6).

MEDs or PNETs represent embryonal tumors of ectodermal origin (7-12). Medulloblastomas may be derived from granule cells of the developing cerebellum. The cerebellar granule cell is the most numerous neuron in the nervous system and is the likely source of medulloblastomas (2). Leung and co-workers showed that Bmi1 is strongly expressed in proliferating cerebellar precursor cells in mice and humans (13). Using Bmi1-null mice, they demonstrated that Bmi1 plays a crucial role in clonal expansion of granule cell precursors both *in vivo* and *in vitro*. Deregulated proliferation of these progenitor cells, by activation of the sonic hedgehog (Shh) pathway, led to MED development. As such, they also linked overexpression of BMI1 and patched (PTCH), suggestive of SHH pathway activation, in a substantial fraction of primary human MEDs. BMI1 overexpression thus serves as an alternative or additive mechanism in the pathogenesis of MEDs. As we reported in one of our articles, this common primary, childhood, cerebellarly located malignancy was named MED by Bailey and Cushing (14) based on the brain developmental theory of Schaper (15), who described the presence of “apolar, indifferent cells in the external granular layer of cerebellum” and named them as “medulloblasts” or the common neural stem cells. Despite a number of morphological, histochemical, and ultrastructural (transmission electron microscopic-TEM and scanning electron microscopic-SEM), and *in vitro* observations, evidence for the real existence of the hypothetical “medulloblast” is still lacking (16). In the great majority of cases, three differentiated cell types are found in childhood MEDs: neurons, glia, and

mesodermal structures (*i.e.* muscle cells). As we reported, because of the presence of multiple differentiated cell types, these tumors were named after a postulated cerebellar stem cell, the "medulloblast", which would give rise to the differentiated cells found in the tumors. A group of researchers at the Massachusetts Institute of Technology (MIT) described a cell line with the properties expected of the postulated medulloblast (17). The rat cerebellar cell line (named ST15A) expressed an intermediate filament, nestin, that is characteristic of neuroepithelial stem cells. ST15A cells can differentiate, gaining either neuronal or glial properties. At the same time several clonal cells can also differentiate into muscle cells. These *in vitro* results suggest that a single neuroectodermal cell can give rise to the different cell types found in MED. Immunocytochemical observations also demonstrated the expression of nestin in human MED tissue and in a MED-derived cell line. Both the properties of the ST15A cell line and the expression of nestin in MED support a neuroectodermal stem cell origin for this childhood tumor. Hart and Earle (18) introduced the concept of PNET, to characterize brain tumors containing 95% or more "small and undifferentiated" cells.

Children with tumors expressing high levels of the neurotrophin-3 receptor, TrkC, have a more favorable outcome (19). During development, TrkC is present in the most mature granule cells. Favorable MEDs may originate from more highly differentiated granule cells. Morphologically MEDs are hypercellular and their microenvironment can be heterogeneous, containing areas of mixed cell populations, neuronal, glial, and/or mesodermal structures. These tumors have the tendency to seed along the cerebrospinal axis and invade the cerebrospinal cavity.

Sixty-three patients with cerebellar MED, treated between 1963 and 1992, were observed by Nishio and co-workers (20). 10 out of 63 patients have survived beyond the Collins' risk period. These included 6 males and 4 females who ranged in age from 6 months to 12 years at the time of diagnosis. A total removal of the tumor was achieved in 4 patients, while there was subtotal removal in 3, and partial removal in 3. Histologically, 6 tumors were classified as classical type MED and 4 were diagnosed as being a desmoplastic type. Postoperatively, 9 patients received craniospinal radiation therapy, and one received local radiation to the primary site. During the follow-up period of 3.9-25.4 years, 5 patients have been in continuous remission for from 14.2 to 25.4 years and are leading normal lives, 2 have survived for 18.1 and 18.5 years with mild to moderate neurological deficits, while the remaining 3 died after the Collins' risk period. Two out of these last 3 patients were under the age of one year at the time of onset, while the remaining one died after a second recurrence. The observations led to the conclusion that that careful follow-up is needed for all long-term survivors even after the Collins' risk period, especially for

those who were under the age of one year at onset and who failed in the initial treatments.

In another study, 27 primary MEDs were analyzed using comparative genomic hybridization and a novel statistical approach to evaluate chromosomal regions for significant gain or loss of genomic DNA (21). An array of nonrandom changes was found in most samples. Two discrete regions of high-level DNA amplification of chromosome bands 5p15.3 and 11q22.3 were observed in 3 of 27 tumors. Nonrandom genomic losses were most frequent in regions on chromosomes 10q (41% of samples), 11 (41%), 16q (37%), 17p (37%), and 8p (33%). Regions of DNA gain most often involved chromosomes 17q (48%) and 7 (44%). These findings suggest a greater degree of genomic imbalance in MED than has been recognized previously and highlight chromosomal loci likely to contain oncogenes or tumor suppressor genes that may contribute to the molecular pathogenesis of childhood MED.

Appropriate prognostic indicators for MEDs in children are of the utmost importance. Immunocytochemical study assessed the prognostic values of N-myc expression in MEDs (22). Nineteen cases of MED or supratentorial PNET (sPNET) were observed for N-myc expression. Sixteen of the observed cases were N-myc-positive, and only three did not express N-myc at detectable levels. N-myc-positive patients had a tendency towards poor disease outcome (p=0.1125). Extended immunohistochemical observations revealed that N-myc-negative tumors were more differentiated towards glial cell lineage than N-myc-positive ones. N-myc-negative and GFAP-positive patients (n=2) tended to survive longer than N-myc-positive and GFAP-negative patients (n=13). The authors concluded that in MED and sPNET patients, N-myc expression may be an appropriate indicator of poor prognosis and more embryonic cell differentiation.

At the beginning of the twenty-first century, no therapeutic regimen can reliably cure PNET. A young age at diagnosis and an advanced stage of the tumor based on the grading of Chang and co-workers should be associated with an unfavorable clinical outcome. The prognostic importance of cell differentiation was addressed with the use of Rorke's classification for PNETs (23, 24), separated into five groups: 1) glial, 2) neuronal, 3) ependymal, 4) multipotential, and 5) without cell differentiation. The cellular classification of brain tumors is based on both histopathological cell and tissue characteristics and location in the brain. Cellular undifferentiated neuroectodermal tumors of the cerebellum have historically been referred to as MEDs, while tumors of identical histology in the pineal region are diagnosed as pineoblastomas. Pineoblastoma and MED are very similar but not identical. The nomenclature of pediatric brain tumors is controversial and potentially confusing (23-35).

The current World Health Organization (WHO) classification groups together both infratentorial neoplasms (MEDs) and their supratentorial counterparts as primitive neuroectodermal tumors (PNETs), implying a common origin. A number of neuropathologists advocate abandoning the traditional morphologically based classifications such as MED in favor of a terminology that relies more extensively on the cell phenotypic characteristics of the tumor. In such a system, MED is referred to as primitive neuroectodermal tumor (PNET) and then subdivided on the basis of cellular differentiation. Nomenclature of neoplasms containing poorly differentiated cells or densely cellular neuroepithelial tumors was simplified to reflect the current state of knowledge of neuroembryology and neuro-oncology, although the authors recognized that such a proposal would likely perpetuate the long-standing and continuing controversy relative to the nature and origin of these neoplasms (23, 24).

The most recent World Health Organization classification of brain tumors still maintains the medical term MED for posterior fossa located, undifferentiated (or dedifferentiated) neoplasms. It also maintains separate categories for cerebral PNETs and for pineal small round cell tumors (pineoblastomas). The pathologic classification of pediatric brain tumors is a specialized area that is undergoing constant evolution (35).

3. GLIAL TUMORS

> "This peculiarity of the membrane, namely, that it becomes continuous with the interstitial matter, the real cement, which binds the nervous elements together and that in all its properties it constitutes a tissue different from the other forms of connective tissue, has induced me to give it a new name, that of neuro-glia (nerve cement)."
>
> - Rudolf Virchow, April 3, 1858 (36)

Malignant childhood ASTRs represent tumors appearing within the neuro-glial or macroglial central nervous system (CNS) (37); they account for over 50% of all intracranial tumors (38-40). Astrocytomas can grow anywhere in the CNS, but in children they usually occur in the brain stem, the cerebrum, or the cerebellum. To be more accurate ASTRs account for about 68 percent of the primary brain tumors occurring in children younger than age 20 (41). The most common brain tumors develop from glial cell precursors (astrocytes, oligodendrocytes, ependymocytes). Glial tumors [mainly astrocytomas (ASTRs)], especially glioblastomas (GBM), are characterized by hypercellularity, pleomorphism, a high number of cell

mitoses, CIP heterogeneity, various grades of necrosis, and multiple endothelial cell proliferations related to newly generated, tumor-related capillaries (see Table II). Furthermore, glial tumors are characterized by high grade local invasiveness and a relatively low metastatic tendency. Cairncross (42) interpreted the histogenesis of ASTRs in the light of parallel concepts emerging from investigations in myeloproliferative disorders (43, 44). According to the stem cell hypothesis, ASTRs originate from a common pluripotent, neuroectodermally derived precursor cell, whose progeny retain the ability to differentiate and do so along astrocytic lines (45). In the last decade it was reported that mutations of P53 gene are present in more than two-thirds of secondary GBMs, but rarely occurs in primary GBMs, suggesting the presence of divergent genetic pathways in their histogenesis (46). The majority of malignant glial tumors are incurable with the current classical therapeutic modalities, including surgical resection, radiotherapy, and chemotherapy (47). This may well be the direct result of the biological variability of these tumors, *e.g.* multiple stem cell lines, intrinsic and acquired multidrug resistance.

The molecular pathogenesis of human ASTRs has been intensely studied during the past few years. Genetic alterations of chromosome 17p are associated with pilocytic ASTRs (World Health Organization (WHO) grade I), mutations at 17p and 19q are common in AAs (WHO grade III) and abnormal chromosomes 17p, 19q and 10 are associated with the most malignant GBM (WHO grade IV) (48). It is well established that low-grade ASTRs have an intrinsic tendency for progressive IP dedifferentiation toward higher-grade, more malignant ASTRs.

The presence of gemistocytes in low-grade ASTRs is regarded as a sign of poor prognosis because the majority of gemistocytic ASTRs rapidly progress to AA or GBM (49). To elucidate the role of gemistocytes in ASTR progression, Watanabe and co-workers assessed the fraction of neoplastic gemistocytes, bcl-2 expression, p53 mutations, p53 immunoreactivity (PAb 1801), and proliferative activity (MIB-1) in 40 low-grade astrocytomas (grade II) with histologically proven progression to AA (grade III) or GBM (grade IV). Astrocytoma progression took significantly less time in patients with a low-grade astrocytoma containing more than 5% gemistocytes (35 months) than in those with lesions containing less than 5% gemistocytes (64 months; p=0.038). All 11 astrocytomas with more than 5% gemistocytes contained a p53 mutation, whereas the incidence of p53 mutations in ASTRs with less than 5% gemistocytes was 61% (p=0.017). In low-grade ASTRs the p53 labeling index of gemistocytes (7.4%) was significantly higher than in all neoplastic cells (3.2%, p=0.0014). Gemistocytes also showed a significantly higher bcl-2 expression than all neoplastic cells, with a mean bcl-2 labeling index of 15.6% vs. 2.7% in low-grade ASTRs (p=0.0004),

20.9% vs. 3.0% in AA (p=0.002), and 30.2% vs. 5.2% in GBMs (p=0.0002). In contrast, in gemistocytes a significantly lower proliferating activity was identified than the mean of all neoplastic cells, with a mean MIB-1 labeling index of 0.5% vs. 2.6% in low-grade ASTRs, 1.5% vs. 11.6% in AA, and 1.7% vs. 16.6% in GBMs (p<0.0001). These data show that low-grade ASTRs with a significant fraction of gemistocytes progress more rapidly and typically carry a p53 mutation. The vast majority of gemistocytes are, however, in a nonproliferative state (G0 phase), which suggests terminal differentiation. Their accumulation within ASTRs may be due to bcl-2 mediated escape from apoptosis.

The prognosis for children with high grade gliomas remains somewhat unpredictable because histologic features alone provide an imperfect assessment of the biologic behavior of a given lesion (50). Whereas some patients experience prolonged disease control after surgery and adjuvant therapy, others with lesions that appear comparable exhibit rapid disease progression and death.

Table 1-1. Histological Types of Childhood Brain Tumors and Their Relative Incidence (after Vats, 1997).

TUMOR	INCIDENCE (%)
Supratentorial	40
Gliomas	21.5
Ependymomas	2.7
Craniopharyngioma	12.4
Infratentorial	54.9
Medulloblastoma (MED)	30.4
Cerebellar astrocytoma (ASTR)	7.3
Ependymoma	53
Brain stem glioma	11.8
Others	4.4

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Chapter 2

IMMUNOPHENOTYPIC CHARACTERIZATION OF INFILTRATING POLY- AND MONONUCLEAR CELLS IN CHILDHOOD BRAIN TUMORS

1. INTRODUCTION

Tumors of the central nervous system (CNS) are poorly responsive to the three classic modality of conventional anti-neoplastic therapy, including surgery, radiation, and chemotherapy. The median survival time of patients treated with surgery alone is 17 weeks which may be extended to 37 weeks through the combination of surgical resection of the tumor mass, radiotherapy, and chemotherapy (1). Infiltration of various CNS tumors by lymphocytes has been observed (2-9).

A fourth, recently developed therapeutic modality in malignant tumor therapy called "adoptive cellular immunotherapy (ACIT)" has been observed to be useful in advanced metastatic, often terminal neoplasm cases. In the majority of cases, the primary tumor and its metastases are infiltrated by a heterogeneous population of poly- and mononuclear leukocytes, including the tumor-associated or tumor-specific antigen-directed cytotoxic T lymphocyte (CTL) clone of the tumor infiltrating lymphocytes (TIL). These "*killer"* cells represent the host's main immunological effector cells and are MHC class I restricted and specifically lyse tumor cell targets. However, the physiologic function of these TILs has yet to be completely understood since they may represent the host's tumor-targeted cellular immune response or simply a cell clone component of a nonspecific inflammatory infiltrate. Immunotherapy has already been employed in various human cancers and

can also be employed in brain tumor cases because tumor infiltrating leukocytes have been observed within the lesions.

The scientific aim of this immunocytochemical study was to characterize the cell surface immunophenotype (IP) of these tumor infiltrating poly- and mononuclear leukocytes with a well characterized library of MoABs directed against cell membrane localized, leukocyte differentiation antigens.

1.1 Results

We observed the expression of various cell membrane located leukocyte cell line differentiation antigens in the leukocyte infiltrates of 76 primary childhood brain tumors, including 34 medulloblastomas/PNETs and 42 astrocytomas. Leu 2/a antigen expression was demonstrated on 58/76 childhood brain tumors establishing the presence of the $CD8^+$, MHC class I restricted cytotoxic T lymphocytes (CTL). These killer cells usually represented 1-10% of all the cells in the tumor frozen section (+), but in some instances constituted 30-44% of all cells (++). $CD4^+$, MHC class II restricted, helper T lymphocytes were present in 65/76 brain tumors and constituted 1-10% of all cells (+). 74/76 childhood brain tumors were infiltrated by macrophages (Leu $M5^+$ cells), and these effectors represented 1-10% of all cells (+) in the tumor frozen section. Of the 76 primary childhood brain tumors observed, 76/76 expressed leukocyte common antigen (LCA), establishing the presence of infiltrating leukocytes. 76/76 pediatric brain tumors also expressed HLA-A,-B,-C and HLA-DR thus demonstrating an MHC class I restriction of the neoplastically transformed cell population as well as further illustrating the presence of subsets and clones of immunological effector cells within the tumor. MoAB UJ 308 detected premyelocytes and mature granulocytes, with unknown functional significance, in 60/76 childhood brain tumors. Natural killer (NK) cells were not defined within any of the tumors we observed.

Solid human tumors are frequently characterized with a markedly heterogeneous poly- and mononuclear cell infiltrate containing macrophages, granulocytes, various subpopulations of T lymphocytes and in some cases such rare cell populations as antibody producing plasma cells and mast cells (10-13). This type of infiltration may vary from florid to none at all, and the phenomenon rarely follows a consistent or predictable pattern.

According to the literature, in our systematic study, we observed some kind of infiltrating leukocytes in 100% (76/76) of the childhood brain tumors cases we examined. We demonstrated the presence of various infiltrating leukocytes on quick-frozen tissue sections in an *in situ* and *ex vivo* manner, thus allowing for a completely accurate observation of the components of the infiltrates as they are in the realm of the tumor (unlike in flow cytometric

analyses where these cells are observed in a culture system and are exposed to a different microenvironment of tissue culture media, bringing about IP alterations (14,15) and a misleading dominance of $CD4^+$ and $CD8^+$ T lymphocytes).

Although immunocytochemical techniques have been previously applied to the study of various primary intracranial tumors, ours is the first report as far as we can determine that not only targeted the two lymphocyte subclasses (cytotoxic and helper), NK cells and possibly macrophages, but also other leukocytes comprising the host's nonspecific immune response such as granulocytes and the MHC restriction of all cells within the brain tumors. Our observations of cell-surface markers present on the various cells comprising the leukocytic infiltrate further clarified our ideas of mechanisms of homing immunological effector cells to the site of the tumor and CTL immunization against various cells among the heterogeneous tumor cell population.

We observed an extremely heterogeneous population of infiltrating leukocytes ranging from the already well investigated $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ helper T lymphocytes to the intriguing presence of premyelocytes and mature granulocytes. We did not observe a predominant presence of neither cytotoxic nor helper T cells, but rather these two types of cells simply represented a small cell clone component of the whole infiltrate. Our observation of no NK cell infiltration at the site of the brain tumors is consistent with observations of rare to slight presence of NK cells in various other brain neoplasms (9, 16). NK cell activity can thus be explained as a nonspecific initial wave of the complete anti-tumor response of the immune system which then gives way to the sustained activity of macrophages, T lymphocytes, and other leukocytic components of the inflammatory infiltrate.

Since 1986 when TILs were first identified as an “anti-tumor” host's cellular response in their functional role by antigen specific (TAA directed) lysis of neoplastic cells (17), many attempts at increasing their efficacy in tumor eradication have been conducted. Lysis of tumor cells is accomplished by a subpopulation of T lymphocytes in the TIL: the $CD8^+$, cytotoxic, MHC Class I restricted T lymphocytes (CTL). In our observation, we established the presence of these killer cells in 58/76 brain tumors. CTL are tumor-targeted, MHC class I restricted, cytolytic cells which also employ specific T cell receptors to mediate their specific anti-neoplastic activities (15, 18-21).

Tumors effectively evade this antigen-specific immune response by down-regulating or losing their cell-surface MHC class I molecules (22). Thus CTL are not reactive with these neoplastically transformed cells. Another very important molecule in tumor-T cell interaction and antigen directed cytolysis is the intercellular adhesion molecule-1. Recently the

immuno-inhibitory shedding of ICAM-1 (soluble ICAM-1) has been identified and has been shown that it inhibits the interaction between T cells and tumor cells. This molecule has been found to be shed by various human melanoma cell lines and binds to the ICAM-1 receptors on T-cells and thus leaves no place for the T cells to bind to the tumor cells' ICAM-1 molecules (23). This further establishes the critical nature of adhesion molecules in mediating intimate cell-cell interactions between various cells at the site of the tumor. It may also present a tumor defense mechanism against antigen-specific lysis by activated T cells.

Another molecule involved in tumor defense mechanisms is the transforming growth factor beta (TGF-β). TGF-β is a tumor derived (autocrine regulation) molecule which has been shown to suppress the *in vitro* generation of CTL from TIL of peripheral blood lymphocytes (24), and thus its *in vivo* secretion at the tumor site could be responsible for the intensive $CD8^+$, cytotoxic T lymphocyte suppression (25,26). Brain tumors have been shown to express various, predominantly low levels of MHC class I molecules as well as ICAM-1 and to produce TGF-β and these observations explain the inability to effectively isolate and expand infiltrating immunological effectors (CTL) from these neoplasias (26). This combination of factors probably represents a common tumor biological phenomenon and apparently renders the infiltrating cells incapable of proliferation and considerably lowers their immunological efficacy. This probably explains the inability of the infiltrating effector cells to overcome tumor progression.

How these cells get to the site of the tumor has been a query long etched in the minds of researchers and several possibilities have been proposed. The most basic is a tumor-specific accumulation of leukocytes brought on by tumor associated or tumor specific antigens. But this explanation has been abandoned for a more general possibility. Site-specific rather than tumor-specific accumulation of these infiltrates has been proposed through observations that leukocytes infiltrating various cutaneous neoplasms express a variety of adhesion molecules such as the integrin, aEb7 (homing receptor), which appears to be involved in the binding of intraepithelial lymphocytes to epithelial cells and the cutaneous lymphocyte-associated antigen, the T cell ligand for E-selectin, located on the surface of endothelial cells, which may mediate the homing of lymphocytes to sites of chronic cutaneous inflammation (27-29).

Our observation of the presence of mature granulocytes among the infiltrating leukocytes in 60/76 tumor cases also substantiates the theory that infiltration occurs due to inflammatory “signals” that cause a nonspecific immune response to occur.

Our ideas concerning the homing of these infiltrates to the site of the tumor have great repercussions in observations concerning the specific immune response to the tumor. Various problems are faced during this response. For instance, the release of many as yet unidentified chemical "radicals" that may well have bearing upon the efficacy of the tumor infiltrating immune effector cell population. During the passage of the neutrophil through the endothelium, numerous cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) are utilized, creating close physical contact between the cells. Proteases and oxygen products (mainly H_2O_2) are released by neutrophils following their activation. The neutrophil-derived H_2O_2 readily diffuses into endothelial cells, triggering a chain reaction that ends in the production of the hydroxyl radical (HO), the toxic oxygen product responsible for endothelial cell injury.

Various inflammatory mediators, including tumor necrosis factor-α (TNF-α), a lymphokine released during the immune response, have been shown to be actively involved in this chain reaction when present at the cite of neutrophils passing through the endothelium (30). Such findings, coupled with our hypothesis of an inflammatory "signal" eliciting a nonspecific immune response to the site of the tumor mass, lead to questions regarding the interaction between the heterogeneous, tumor infiltrating mononuclear cell mass and the endothelium through which they must pass in order to reach the tumor cell mass. What is the nature of the chemicals released during the interaction between immune effector cells and endothelial cells and do these as yet unidentified chemicals reduce the efficacy of these effector cells against the tumor?

In view of our results, we propose that the leukocytic infiltrate, comprised of various immune effector cells including TIL, is first attracted to the tumor site as part of a nonspecific immune response to an inflammatory "signal" or necrotic transformations in the tumor mass. Furthermore, following these necrotic changes in the neoplastic cell mass, monocytes/macrophages, acting in their antigen presenting cell role, consume the necrotic cells and present previously "hidden," tumor-associated and tumor-specific antigens (TAAs and TSAs) to the other effector cells in the infiltrate, thus establishing immunization against the tumor mass as a process which occurs *in situ.* Thus, the infiltrating leukocytes are in a "developmental" stage when they first arrive at the tumor site, and this development begins with the initial nonspecific reaction and evolves into a specific reaction following *in situ* immunization.

Neoplastically transformed cells undergo constant microevolution. Natural selection of the most advantageous surface IP involves constant modulation of previous IPs. Progressive dedifferentiation characterizes all neoplastically transformed cells. During this process, numerous "novel" cell

surface antigens appear, are modified and thus do present the host's immune system with some immunogenic elements. The leukocytic inflammatory infiltrate contains cells with diverse capabilities including neutrophils, macrophages and DCs as professional antigen presenting cells (APCs), as well as T lymphocytes. *In situ* activation of TAA specific cytotoxic T lymphocyte (CTL) clones occurs and thousands of neoplastic cells are lysed. However, as we would expect from any population in danger of extinction, the cells of the neoplastically transformed mass proceed with their microevolution and numerous clones of tumor cells survive each repeated attack by the immune system through secretion of immuno-inhibitory cytokines, such as TGF-β which has both ECM modulatory and direct suppressive effects on CTL generation from peripheral blood lymphocytes (24,25,31,32), downregulation of MHC molecules (26,33), loss of adhesion (34) and costimulatory molecules and induction of clonal T cell anergy (23,35), among other as yet uncovered ways. This process continues until the "creation" (ironically as it may sound, by the host's immune system) of highly resistant, poorly immunogenic, and extremely aggressive clones of tumor cells. This is the reality of cancer progression: a back-and-forth struggle between host and tumor, with evolutionarily dynamic exchanges throughout the entire process.

The expression of apoptosis related cell surface molecules on the surface of both tumor cell and CTL surfaces (*FasR-FasL* system) raises a distinct possibility of active PCD induction in CTL by tumor cells. Juxtacrine interactions between CTL and neoplastically transformed cells, coupled with observations that tumor cells can modulate the intracellular, signaling domains of cell surface receptors to elicit responses quite often contrary to the expected, may even provide a way for CTL to enhance the proliferation and dedifferentiation of cancer cells. Adoptive therapies using CTL raised against autologous neoplastically transformed cells *in vitro* should be employed in the control of minimal residual disease following surgical resection of the primary malignant growth. Further studies should establish the clinical significance of PCD-related protein expression and assess the possibility of targeting such molecules in the therapy of human neoplasms.