Abstract. The overexpression of COX enzymes has been demonstrated in human neoplasms at various sites, including the colon, gastrointestinal tract, lung, skin and recently in brain tumors. In this study, COX-2 receptor overexpression in primary childhood brain tumors was determined and the distribution pattern of COX-2 receptors was examined. A sensitive, 4-step, alkaline phosphatase conjugated antigen detection technique was used and a specific monoclonal antibody for medulloblastomas/primitive neuroectodermal tumors (MEDs/PNETs), anaplastic, high-grade astrocytomas (ASTRs) and in glioblastoma multiformes (GMs) was employed. All of the 14 MEDs/PNETs observed demonstrated high levels of immunoreactivity (overexpression), with the highest immunostaining intensity (grades A and B). However, of the 14 subtypes of astrocytic tumors examined, the COX-2 receptor expression level did not even approach those of the MEDs/PNETs levels. However, significant differences were found when comparing low grade pilocytic ASTRs to high grade anaplastic ASTRs and glioblastomas. In two low grade pilocytic ASTRs, the expression level never exceeded 20%, while in high grade glial tumors (6 anaplastic ASTRs and 6 GMs) 30 to 50% of the tumor cells overexpressed COX-2 receptors, documenting an increase in COX-2 receptor overexpression with the increasing grade of the astrocytic tumor. In view of these findings, it would appear likely that COX-2 inhibitors may represent a chemo-preventive tool in treating childhood brain tumors, which are the leading cause of solid tumor cancer death in children under the age of 20.

Both prostaglandin (PG) synthases, also called cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), are catalytic enzymes involved in PG synthesis from arachidonic acid (1-4). Prostaglandin E2 (PGE2) functions promote the primary process of carcinogenesis and its further consolidation and progression via increased cell proliferation, decreased natural killer cell activity, in situ immune down-modulation, induction of neo-angiogenesis and the elevated expression of anti-apoptotic protein Bcl-2 (5). In addition, PGE2 significantly alters immune surveillance. COX-2, a principal COX isoenzyme, is induced in response to the actions of growth factors, various cytokines and promoters of neoplastic cell transformation and progression (4, 6, 7).

An overexpression of COX enzymes has been demonstrated in human neoplasms at various sites, including the colon, gastrointestinal tract, lung, and skin (8). Immunohistochemical analyses of COX-2 expression identified that it is elevated in up to 90% of sporadic colon carcinomas and 40% of colonic adenomas (9, 10); it is also overexpressed in 40% to 80% of malignancies of the lung, head and neck, breast, prostate, brain and pancreas (11-13). Increased levels of COX-2, prostaglandins, or both, are found in adenomas in patients with familial adenomatous polyposis (14).

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the immunohistochemical techniques employed in this study have unequivocal prognostic value (22, 24-26).

Hart and Earle introduced the concept of PNET to describe a series of supratentorial tumors that contained 95% or more of small undifferentiated cells not referable to the then current classification (27). No implication was made of a common neural stem cell origin, nor was there a hypothesis of developmental stage-dependent expression of antigens. Rorke (28, 29) called the PNET a "wastebasket category" and proposed a revised World Health Organization (WHO) brain tumor classification based upon the intratumoral grade and type, as well as the main direction of cytomorphological differentiation (30). Rubinstein, however, expressed doubt about the common tissue origin and bracketing of infratentorial and supratentorial PNETs (sPNETs), such as MED, ependymoblastoma, central neuroblastoma, pineoblastoma (31), which are all referred to as PNETs according to the proposed classification (30).

Morphologically, both MEDs and sPNETs are hypercellular and can be heterogeneous, containing areas of mixed cell populations (32, 33), neuronal (34-38), glial (36, 38-41) and/or mesodermal structures (42).

Glial tumors [mainly astrocytomas (ASTRs)] and especially glioblastomas are characterized by hypercellularity, pleomorphism, a number of cell mitoses, CIP heterogeneity, various grades of necrosis and multiple endothelial cell proliferations related to the newly generated, tumor-related capillaries (43-65). Furthermore, glial tumors are characterized by high grade local invasiveness and a relatively low metastatic tendency.

The specific aims of the present immunohistochemical study were: i) to detect overexpression of COX-2 receptors in primary childhood brain tumors; and ii) to observe the distribution pattern of COX-2 receptors.

Materials and Methods

**Tissues and tissue handling.** In this immunohistochemical study, the following formalin-fixed, paraffin wax-embedded tissue sections of human primary childhood brain tumors were used: i) MEDs/PNETs and ii) different subtypes of ASTRs, all purchased from DAKO Corporation (Carpinteria, CA, USA). The diagnoses of the specific subtypes of the gliomas, observed in this study were established according to WHO guidelines for the classification of gliomas by a clinical neurohistopathologist (66-70). The technical details of the immunohistochemical techniques employed in this study have already been elaborated by other investigators (71-78) and in the immunohistochemical studies published by our group (79-85).

**Monoclonal antibody.** Mouse anti-COX-2 (Cat. #35-8200; Zymed Laboratories, Inc. South San Francisco, CA, USA), clone; COX 229, Isotype: Mouse IgG1-k was employed.

Staining of formalin-fixed and paraffin wax-embedded tissue sections required an antigen retrieval technique, employing preliminary boiling in 10 mM citrate buffer, pH 6.0 (NeoMarkers, Cat. # AP-9003) for 10-20 min, followed by cooling at room temperature for 20 minutes.

**Immunohistochemical controls.** In order to ensure the specificity of the antibody employed in this study, the immunoreactivity of several normal human control tissues including, brain, adrenal, heart, stomach, small intestine, large intestine, liver, kidney, pancreas, lung, testis, ovary, prostate, thyroid and spleen, all included in one checkerboard multitissue block (DAKO Corporation, Carpinteria, CA, USA; code # T1065) were tested (86, 87). Several postnatal human thymic specimens were also used as negative and positive tissue controls. A number of neoplastically transformed tissues, including malignant melanoma and lung cancer tissues, also represented positive tissue controls. Additional controls for all tissues and MoABs included: i) omission of the primary antibody; ii) utilization of only the enzymatic developer solution to detect the presence of endogenous alkaline phosphatase activity; and iii) utilization of MOPC 21 mouse myeloma IgG1 (ICN) as a replacement for the primary MoAB to determine non-specific myeloma protein binding to the antigen epitopes of the screened tissues.

**Immunoalkaline phosphatase antigen detection technique.** We used the following immunoalkaline phosphatase cytochemical method (71-78), modified, for antigen detection in formalin-fixed, paraffin-wax embedded anaplastic ASTR and glioblastoma tissues. The technique is a highly sensitive, indirect, four-to-six-step immunocytochemical method, which combines the biotin-streptavidin-based ABC-method with enzyme-linked (alkaline phosphatase-AP) immunohistochemistry and has been previously described in detail (79-88). Briefly, following deparaffinization in three changes of Xylene substitute (Shandon-Lipshaw, Pittsburgh, PA, USA) for 20 to 30 min, rehydration was carried out employing descending dilutions of alcohol (100% to 50%) to TBS. An initial blocking step using 1% glacial acetic acid mixed with the working buffer for 10 minutes was necessary to eliminate the endogenous AP activity from the tissues. The use of levamisole solution is also described in our earlier observations. As previously described (79-88), GAA inhibition was preferred because of the possible presence of levamisole-resistant AP isoenzyme (73). The second blocking step was conducted with a purified mixture of proteins (Shandon-Lipshaw) from various species for 5-10 min to block cross-reactive antigenic epitopes. Excess serum was removed from the area surrounding the sections. The tissue sections were then incubated for 60-120 min with the COX-2 primary antibody. Next, incubation with the secondary antibody, goat anti-mouse IgG molecule (IgG molecule diluted by ICN Biomedicals, Inc., Aurora, OH, USA), was carried out for 20 min. Streptavidin conjugation was accomplished by incubation with AP conjugated streptavidin for 20 min (75-78). Color visualization of the primary antigen-antibody (Ag-Ab)
reaction was accomplished with an alkaline phosphatase (AP) kit I (Vector Laboratories, Burlingame, CA, USA), which contains AS-TR with Tris-HCl buffer at pH 8.2, added for 28-40 min to allow formation of a stable red precipitate. Sections were counterstained with a diluted solution of Gill’s hematoxylin (Richard-Allan, Kalamazoo, MI, USA). The tissue slides were then dehydrated in ascending concentrations of alcohol (60% to 100%) to xylene substitute (Shandon-Lipshaw), in which they were kept overnight to ensure complete morphological clearing. The stained tissue sections were mounted using a solution specially designed for use following morphological clearing in xylene substitute (Shandon-Lipshaw).

**Immunohistochemical evaluation.** A qualitative and quantitative evaluation of the percent of antigen-positive cells and the intensity of IGF immunostaining were conducted employing a light microscope (Olympus, Japan) to count 100-200 cells. Artifacts were avoided, while, on the other hand, morphologically characteristic areas were sought out. The presence of brain tumor cells and the endothelial elements of small blood vessels, tumor infiltrating leukocytes and macrophages (the host’s immunological effector cells) required careful qualitative assessment. Non-vascular elements were also examined, but only morphologically distinct brain tumor cells were scored.

**Quantitative evaluation (88):** The following evaluations were used:
- (+ + + +) over 90% of the total brain tumor cells are positive;
- (+ + +) 50% to 90% of the total tumor cells are positive; (+ +) 10% to 50% of the total tumor cells are positive; (+) 1% to 10% of the total brain tumor cells are positive; (±) under 1% of the total tumor cells are positive; (–) negative.

**Qualitative evaluation (88):** The following scores were used:
- (A) very intense red staining;
- (B) strong red staining;
- (C) light red staining;
- (D) negative staining.

**Results**

In our systematic immunohistochemical screening, the overexpression profile of COX-2 receptors was observed in 14 MEDs/PNETs and 14 subtypes of astrocytic tumors. All 14 MEDs/PNETs observed demonstrated high levels of immunoreactivity (overexpression), detectable by the highest immunostaining intensity grades A and B (Figure 1). In the MEDs/PNETs, 30 to 50% (+ +) of the tumor cells demonstrated strong presence of the COX-2 receptor. In the observed ASTR subtypes, the COX-2 receptor expression level demonstrated significant differences between the low-grade pilocytic ASTRs and the high-grade anaplastic ASTRs and glioblastomas. In two low-grade pilocytic ASTRs, the expression level never exceeded 20% and was usually between 5 to 15% (+). On the contrary, in high-grade glial tumors (6 anaplastic ASTRs and 6 GMs) 30 to 50% (+ +) of the tumor cells overexpressed COX-2 receptors.

**Discussion**

It is well established that dysregulation and altered functioning of various molecular biological processes and signaling pathways are involved in the development and progression of malignant neoplasms, including brain tumors (89). Employment of chemotherapy in combination with radiotherapy has emerged a strategy of choice in the antineoplastic treatment of advanced human neoplasms (90).

COX-2, the isoform of the enzyme cyclooxygenase that is inducible by cytokines, mitogens and growth factors (91), has become one of the principal targets of current antineoplastic chemotherapies. COX-2 is often overexpressed in premalignant and malignant lesions and usually signals a more aggressive pathobiological tumor behavior. The results of the present immunohistochemical study confirm the overexpression of COX-2 in primary human childhood brain tumors, as well as its differential expression in ASTR subtypes. COX-2 has been shown to induce resistance in neoplastically transformed cells to chemotherapeutic agents or radiation. In vitro experimental systems have revealed that selective COX-2 inhibitors cause a decrease in cell proliferation, an increase spontaneous cell death (apoptosis) and modulate cell cycle regulations at gene levels. Molecular targeting the COX-2 enzyme with selective inhibitors was found to be a potent enhancer of neoplastic radioresponse in several preclinical models (92). In fact, COX-2 inhibitors that have been studied in cancer chemoprevention are now under active investigation for cancer therapy (93). In order to maximize the benefit of COX-2 inhibitor therapy in the clinical setting, it is important to investigate the role of molecular targets of such therapy both in established human cancers and in pre-neoplastic lesions, as well as in its effects on currently used therapeutic modalities such as radiotherapy (94).

Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) are the most widely studied agents for the chemoprevention of several human malignancies (95, 96). These compounds exert their effects by a number of mechanisms. They inhibit both COX-1 and COX-2 (97). When treated with sulindac, an NSAID that inhibits both COX-1 and COX-2, the number of intestinal adenomas is reduced by more than 90% (98) and the total volume of colon tumors by more than 52% (99). This reduction in polyp formation can be improved by use of a more selective COX-2 inhibitor or by the deletion of the COX-2 gene itself (100, 101). The mechanisms by which the inhibition of COX-2 leads to decreased colon carcinogenesis are not fully understood, but may involve an increase in apoptosis (101, 102), the regulation of angiogenesis, or both (103, 104). Aspirin and other NSAIDs may also act by COX-independent mechanisms, such as inhibition of the activation of nuclear factor-κB (NF-κB) (105) or interference with the binding of the peroxisome-proliferator-activated receptor (PPAR) to DNA (106).

A selective COX-2 inhibitor, celecoxib, was shown in a randomized study to cause regression of polyps in patients with familial adenomatus polyposis. Celecoxib significantly reduced the number of colorectal polyps by 28% after 6 months of treatment, compared with patients administered
placebo (107). Celecoxib currently is being investigated in trials for Barrett’s esophagus and cancers of the bladder, colon, mouth, prostate and skin (108).

Sulindac and its derivatives have demonstrated antineoplastic effects—through COX-1, COX-2 and LOX inhibition (109). The LOX pathway in AA metabolism is a promising target for chemoprevention research; LOX inhibition has been shown to reduce tumor cell growth more effectively than COX inhibition (110). LOXs have been associated with various neoplastically transformed cell types, including breast, colon, prostate, pancreas and non-small-cell lung cancers (111, 112).

In conclusion, inhibitors of the cyclooxygenases may be yet another component to be considered when developing the "cocktail" that will lead to the most efficacious treatment of neoplastic disease. Based upon our results, the COX-2 receptor may now be added to the long list of brain tumor progression markers and possible therapeutic targets.

References


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