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Toll-like receptor 2 (TLR2) is a marker of angiogenesis in the necrotic area of human medulloblastoma

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Abstract

Angiogenesis plays a key role in the progression of malignant tumors. In recent years, anti-angiogenic drugs have been shown to be effective against tumors. However, some tumors are able to adopt escape mechanisms, suggesting that the vascular network in these tumors may be formed or may function in a different way. Medulloblastomas are tumors characterized by poor prognosis and low patient survival rates. These tumors rarely metastasize, but the reason why they almost always recur locally is not known. Central to mediating neoplastic changes is the interaction between cell surface receptors and their cognate ligands, which through intracellular signaling induce alternations in gene expression. In this context, the aim of our present study was to examine in medulloblastoma the distribution of Toll-like receptor 2 (TLR2) and receptor for advanced glycosylation end-product (RAGE), and mast cells associated with the tumor neovascularization process. Immunohistochemical study with a battery of specific antibodies was used. The results show that in the tumor necrotic area, TLR2 participates in all steps of vascular network formation, but in regions where the tumor was not affected by necrosis, the capillary network was TLR2 immunonegative. The TLR2 vascular network of the necrotic area was not associated with RAGE and mast cells. However, in the region of the medulloblastoma not affected by necrosis, the RAGE receptor was present in the endothelium of all capillaries, and mast cells were numerous only in the perivascular space of large brain and meningeal vessels at the border of the tumor. In conclusion, our results show that the receptor of innate immunity TLR2 plays an important role in recognition of ligands delivered by dying necrotic medulloblastoma cells and participates in tumor neovascularization. Moreover, the results show that the RAGE receptor and mast cells operate in different medulloblastoma regions and influence different parts of the tumor vascular network.

Key words: *medulloblastoma, receptor for advanced glycosylation end-product (RAGE), mast cells, tumor angiogenesis, Toll-like receptor 2 (TLR2).*

Introduction

The development of the vascular network plays an essential role in physiology and pathology. Three mechanisms – vasculogenesis, angiogenesis and arteriogenesis – participate in this process. Vasculogenesis occurs by angioblast differentiation into endothelial cells to form blood vessels. Angiogene-

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Danuta Maslinska, Milena Laure-Kamionowska, Sławomir Maslinski, Dariusz Szukiewicz

sis is the process in which new blood vessels are formed from pre-existing ones, and arteriogenesis refers to the enlargement of the arterial network to sustain higher metabolic demands of the tissue [19]. In oncology, tumor vascularity is correlated with tumor growth, invasion and initiation of metastasis [2] and is thought to occur mainly by angiogenesis [10], although the role of postnatal vasculogenesis by recruitment of bone marrow-derived endothelial progenitor cells (EPCs) into the tumor vasculature has also been discussed [9,27]. Formation of a new capillary network involves many steps of a cascade in which one event triggers the next through the action of specific mediators. These mediators belong to different sets of factors participating in tumor angiogenesis. One of the sets includes numerous pro-inflammatory mediators such as growth factors, cytokines, proteoglycans and lipid mediators, but all of them are delivered from any cell injured by hypoxia, ischemia, free radicals or infection agents. The other, recently studied, set of angiogenic mediators comprises those that are fragments of destroyed host necrotic cells. Such mediators were recognized as the damage-associated molecular pattern (DAMP) molecules that have been implicated in several inflammatory diseases and activate target cells through the differential engagement of multiple surface receptors including innate immune receptors such as Toll-like receptor 2 (TLR2), or receptor for advanced glycation end-products (RAGE). In addition, mast cells, which are abundant surrounding solid tumors, are a source of numerous compounds that are often suspected of participation in tumor angiogenesis. Recently, we observed that TLR2 is a very sensitive marker of a newly formed tumor capillary network in medulloblastoma [28].

Medulloblastoma is the most common primary solid brain tumor occurring in children, with a strong male preponderance. Histologically, the tumor is composed of sheets or lobules of small cells with prominent nuclei and little cytoplasm. Vascular proliferation may be present but is not a constant feature of this lesion, but as in other tumors neovascularization is an important mechanism underlying medulloblastoma progression. Increased tumor angiogenesis is normally associated with poor prognosis of the disease, and the development of therapeutic agents that can reduce or inhibit angiogenesis or destroy the neovasculature of tumors has recently received considerable interest [31]. One possible advantage

of these drugs, compared with conventional chemotherapy, is their potential for reduced susceptibility to acquired drug resistance. This is a major clinical problem observed in patients undergoing chemotherapy and results from the intrinsic genetic instability and heterogeneity of tumor cells [29]. Endothelial cells, on the other hand, on which anti-vascular and anti-angiogenic drugs act, are genetically more stable and more homogeneous and are thus presumed to be not so susceptible to acquired drug resistance. Our growing understanding of the molecular mechanisms underlying angiogenesis, including the role of several growth factors that regulate the process, has enabled the development of a large number of drug candidates against vascular-specific targets that can inhibit angiogenesis.

Since there are numerous unanswered questions concerning the process of angiogenesis which occurs in the pathological microenvironment of cancer, the aim of our present study was to examine in medulloblastoma of the human brain the distribution of TLR2 and RAGE receptors and mast cells associated with the tumor neovascularization.

Material and methods

The study was approved by the local ethical committee and performed on human brains that were obtained following autopsy. Patients died at different ages of life (from 1-5, 17, to 37 years of age), one to two years after ineffective therapy. Brains with medulloblastoma were diagnosed according to the WHO classification [26] in the Institute of Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland. Paraffin blocks were drawn from the file, cut into serial sections (5 µm thick) and stained with H&E for routine histological examination or used for immunohistochemical study.

Immunohistochemistry

Immunohistochemistry was performed using specific primary antibodies and an alkaline phosphatase-avidin-biotin conjugate purchased from Santa Cruz Laboratory (USA) or an avidin-biotin-peroxidase complex system purchased from Vector Laboratories (USA) and used according to the manufacturer's recommendations.

Briefly, sections were dewaxed and hydrated through descending alcohols to water. For non-enzymatic antigen retrieval, some sections were heat-

Table I. Antibodies used in the study

Antibody	Type	Antigen		Source	Dilution
		Origin	Domain		
TLR 2	Polyclonal	Human	*N*-terminus	Santa Cruz	1 : 50
TLR 4	Polyclonal	Human	*C*-terminus	Santa Cruz	1 : 50
MC tryptase	Polyclonal	Human	Not quoted	Chemicon,	1 : 100
RAGE	Polyclonal	Human	*C*-terminus	Santa Cruz	1 : 50
RAGE	Polyclonal	Human	*N*-terminus	Santa Cruz	1 : 50

ed in 0.01 M sodium citrate buffer (pH 6.0) to 95°C and allowed to cool for 20 min at room temperature and washed with PBS. Then, they were incubated in methanol/3% H_2O_2 solution for 20 min to quench endogenous peroxidase. After being washed again in PBS and blocked with solution containing PBS/5% appropriate normal serum (of goat, rabbit or mouse) for 2 h at room temperature, sections were incubated overnight at 4°C in solutions of primary antibodies listed in Table I.

Immunoreactions were visualized using biotinylated secondary antibodies and ABComplex/HRP or an alkaline phosphatase-avidin-biotin conjugate. Then, sections were lightly counterstained with Mayer's hematoxylin.

For negative controls, primary antibodies were replaced with an appropriate isotypic normal mouse, rabbit or goat immunoglobulin fraction at a matched protein concentration. These were included for the examination of each specimen and consistently produced negative results.

Results

Distribution of the TLR2 capillary network, mast cells and RAGE in different regions of medulloblastoma is presented in Table II.

In medulloblastoma of all our patients, neovascularization/angiogenesis (V/A) was observed in areas of tumor cell necrosis. We observed the successive steps of this process. Cells participating in each such step were immunopositive to TLR2. At the beginning, TLR2 immunopositive cells were scattered throughout the tumor necrotic debris (Fig. 1A). In the next step, they formed tubular structures (Fig. 1B) which penetrated to the post-necrotic areas already cleared of dead cells (Fig. 1C). Finally, numerous TLR2-immunopositive capillaries formed a very dense vascular network which closely conforms to the post-necrotic space of the tumor (Fig. 2). In the capillary wall of this network RAGE receptors were not found. In addition, mast cells were not detected in these necrotic or post-necrotic areas of the tumor.

In non-injured regions of the medulloblastoma, the vascular network was immunonegative to TLR2, but RAGE protein was precisely localized in the wall of all capillaries and in proliferating endothelium of some of these vessels (Fig. 3A-B).

Mast cells were distributed at the border of the medulloblastoma and were localized at the perivascular space of large brain vessels (Fig. 4A). Those mast cells that were detected within the solid tumor regions belonged to the host meninges that were incorporated into the tumor mass (Fig. 4B).

Discussion

Reciprocity of inflammation and neovascularization is emerging as an important mechanism underlying numerous processes from tissue healing/remodeling to cancer [3,17].

In recent years, anti-angiogenic therapies have been developed and tested for their effectiveness against tumors. Among such drugs are inhibitors against vascular endothelial growth factor and its receptors, as well as inhibitors targeting the plate-

Table II. Distribution of Toll-like receptor 2 (TLR2) capillary network, mast cells and receptor for advanced glycosylation end-product (RAGE)

	Medulloblastoma regions	
	Post-necrotic areas	Solid tumor areas
TLR2 capillary	Dense network	nd
RAGE	nd	Capillary endothelium
Mast cells	nd	Perivascular area

nd – not detected

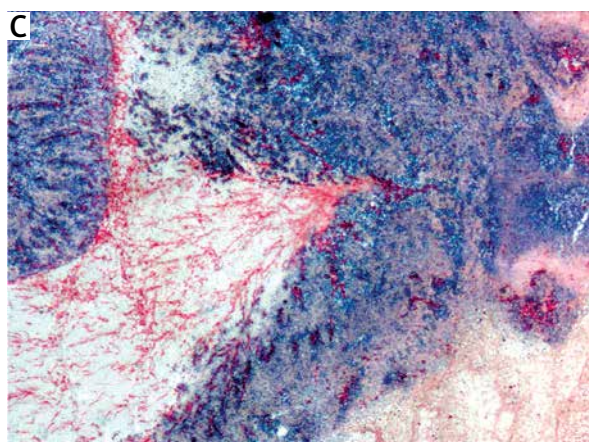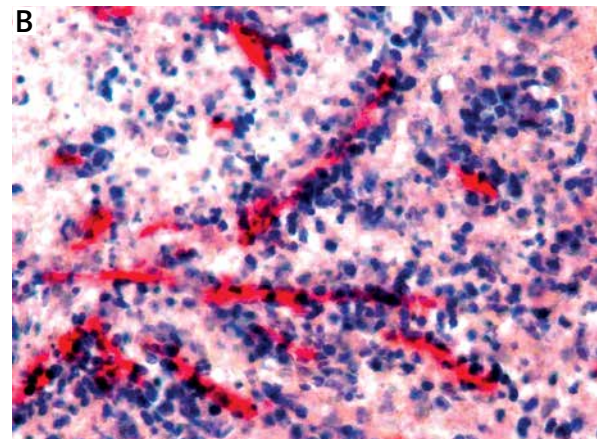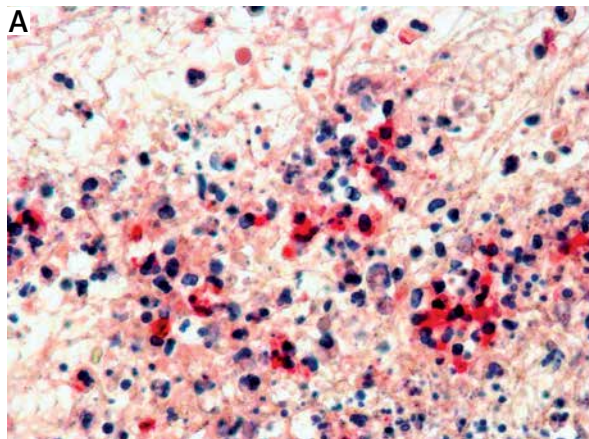Danuta Maslinska, Milena Laure-Kamionowska, Sławomir Maslinski, Dariusz Szukiewicz

Fig. 1. Steps of capillary network formation by Toll-like receptor 2 (TLR2) immunopositive cells in necrotic area of medulloblastoma. **A)** TLR2 immunopositive cells scattered throughout the tumor necrotic debris; magn. ×200. **B)** TLR2 immunopositive tubular structures; magn. ×200. **C)** TLR2 immunopositive capillaries infiltrating post-necrotic areas; magn. ×100.
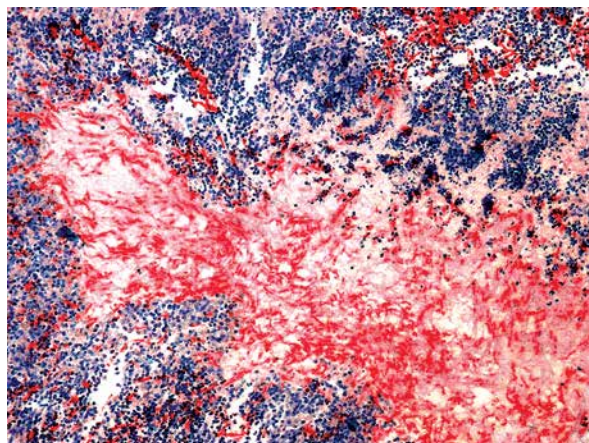

Fig. 2. Toll-like receptor 2 (TLR2) immunopositive vascular network closely conforms to post-necrotic area of the tumor; magn. ×100.

let-derived growth factor family, integrins or histone deacetylase. While many anti-angiogenic drugs have been shown to be effective with limited toxicity, some tumors are able to adopt escape mechanisms [24], suggesting that the vascular network in some tumors could be formed in a different way than normal capillaries. The results of our present study confirm this hypothesis. In regions of medulloblastoma cell necrosis, we observed that TLR2 participates in all steps of vascular network formation, but in regions where the tumor was not affected by necrosis, the capillary network was TLR2 immunonegative. We believe that all those TLR2 immunonegative capillaries were developed during normal host embryogenesis and the space supplied by this capillary network was colonized by medulloblastoma cells. Thus, only in necrotic areas of the medulloblastoma were pathogens delivered from dying undifferentiated tumor cells recognized by TLR2 and activated TLR2 signaling promoted the angiogenic responses in the tumor.

Toll-like receptors (TLRs) are a family of pattern recognition receptors that have emerged as key mediators of innate immunity. These receptors recognize a broad range of protein and lipid ligands [21,33], including a number of host origin ligands [1,18,22,36,37,41], and initiate host defense inflam-
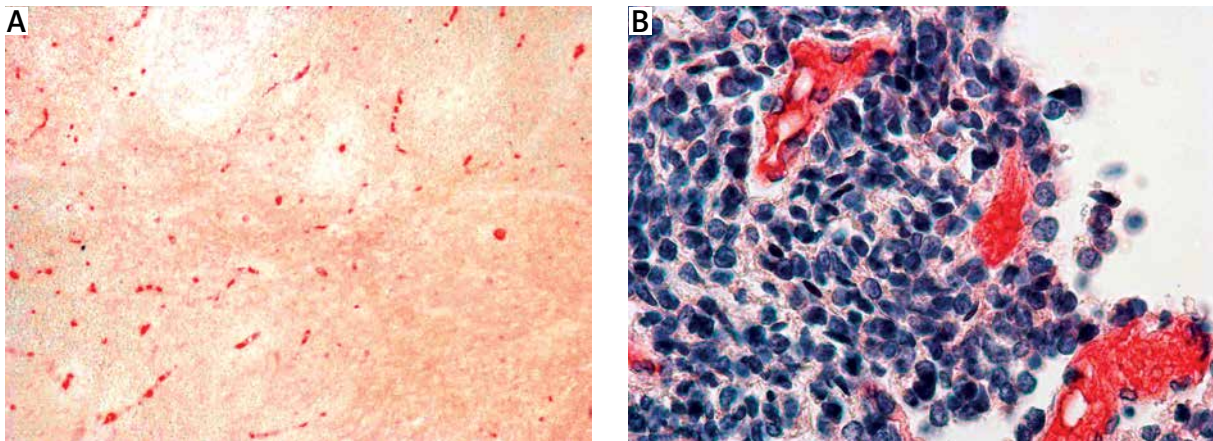
Fig. 3. Receptor for advanced glycosylation end-product (RAGE) immunopositive capillaries in non-injured tumor regions. **A)** RAGE in capillaries (section not counterstained); magn. ×100. **B)** RAGE in proliferating endothelium of tumor blood vessels; magn. ×200.
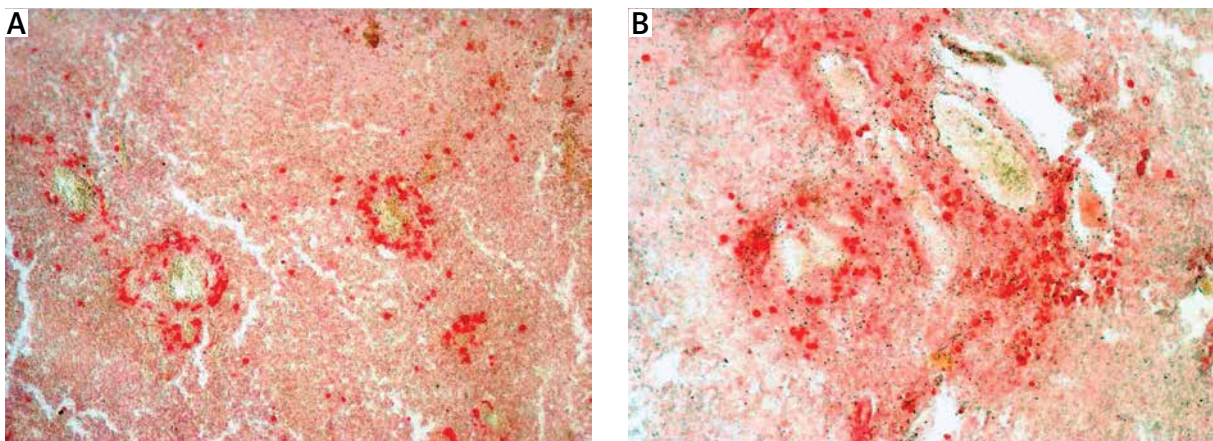

Fig. 4. Tryptase immunopositive mast cells in perivascular space of brain large blood vessels (sections not counterstained). **A)** At the border of medulloblastoma; magn. ×200. **B)** In blood vessels of meninges; magn. ×200.

mation. TLR-mediated inflammation is an important pathogenic link between innate immunity and a diverse panel of clinical disorders including cancer. In addition, expression of TLR2 and TLR4 was found on the endothelium; therefore both receptors, in some circumstances, may be implicated in angiogenesis [8].

Altogether our results indicate that TLR2 is a sensor of pathogens of necrotic tumor cells, which provides a key link connecting innate immunity and cancer angiogenesis.

Tumorigenesis is a multi-step process involving the alternation of a number of key cellular properties including uncontrolled proliferation, evasion of cell death (apoptosis), vascularization (angiogenesis) and subsequent invasion and migration of tumor cells into the surrounding tissues [10]. Understand-

ing the molecular processes underlying these cellular phenotypic changes is critical in order to develop novel therapies. Central to mediating these changes is the interaction between cell surface receptors and their cognate ligands, which through intracellular signaling induce alternations in gene expression. In this context, recent studies have identified that the receptor for advanced glycation end-products (RAGE) and its ligands may play an important role in cancer [7,25,38,39]. RAGE is a multi-ligand receptor that has been implicated in the pathogenesis of numerous diseases [20,38,42]. RAGE-ligand interaction triggers activation of a diverse array of signaling pathways that lead to processes integrally linked to the tumorigenic sequelae including cellular migration, invasion, proliferation and survival [25,29]. Furthermore,

Danuta Maslinska, Milena Laure-Kamionowska, Sławomir Maslinski, Dariusz Szukiewicz

studies have indicated that RAGE and its ligands are expressed in human tumors, and often the extent of tumor invasiveness and metastatic potential was correlated with the degree of RAGE/ligand up-regulation [11-16,23,30,35,40].

Brain compartments are effectively isolated from the plasma proteins and various ligands by the blood-brain barrier (BBB), localized on the endothelium of the brain capillaries. However, at this barrier, there are specialized receptors including RAGE that may shuttle all such proteins in efflux and influx directions [4,5]. The results of our present study suggest that RAGE does not participate in the blood-brain function of the capillary network formed in post-necrotic regions of the medulloblastoma and ligands of this receptor cannot affect the healing process of the tumor. However, in all other regions of the tumor, RAGE is present on capillary endothelium, and thus the blood-brain barrier functions differently than in post-necrotic areas. In addition, mast cells, known as a source of early-response cytokines decisive in initiating the immune and host defense reaction to pathogens, were not found in the vicinity of capillaries in any regions of the tumor. Despite many reports concerning the association of mast cells (MC) with a variety of tumors, the functional significance of these cells in tumor biology remains obscure. The presence of mast cells has been interpreted as an immunological anti-tumor response or as a more direct interaction with tumor cells, especially an interaction which facilitates tumor growth [32]. The results of our study suggest that mast cells have little if any influence on development of angiogenesis in post-necrotic regions of the medulloblastoma.

Mast cells are a heterogeneous cell population categorized as either mucosal type or connective tissue type, based on their tissue locations, staining patterns, content of proteases and reactivity. In humans, mature mast cells in the brain contain tryptase as a protease and are known as the MC tryptase phenotype (MCT) [34]. Mast cells provide different multifunctional cytokines and potent mediators can mediate a variety of antimicrobial functions and possess certain unique features that make MC function particularly crucial for host defense [6]. Mast cells are always located at the host-environment interface, and they belong to the first line of cells that encounter pathogens. In our study, we found many of these cells at the border of the tumor and in meninges that covered the tumor. All MC were located at the perivascular space of large blood vessels. Therefore we conclude that MC were not directly involved in the function of the blood-brain barrier or in the process of angiogenesis of medulloblastoma post-necrotic areas. However, MC are long-lived cells with the capacity to respond repeatedly to the same stimulus, and we suspect that in our patients they had a potent influence on the function of the vascular network at the peripheral regions of the tumor.

Medulloblastomas are characterized by poor prognosis and low patient survival rates. In particular, strong induction of angiogenesis marks the transition from a lower-grade medulloblastoma to a more aggressive and lethal form of the tumor. Although these tumors rarely metastasize, they almost always recur locally. Data from our study suggest that this may be linked with angiogenesis and the healing process in medulloblastoma necrotic areas.

Despite advanced clinical approaches with surgery, radiotherapy and chemotherapy, inhibition of angiogenesis might represent a key strategy in the treatment of this neoplasm.

Nevertheless, the success with existing compounds in the managements of brain tumors is limited, and further study is required. We present new data focused on tumor angiogenesis. They show that capillary network formation and function of the blood-brain barrier differ depending on the medulloblastoma region and that receptors of innate immunity play an essential role in these processes.

Disclosure

Authors report no conflict of interest.

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