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Original Contribution

Expression of the Annexin A1 and its correlation with matrix metalloproteinases and the receptor for formylated peptide-2 in diffuse astrocytic tumors☆

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ABSTRACT

Astrocytomas represent the majority of cerebral gliomas. Studies show that the anti-inflammatory protein Annexin-A1 (ANXA1) is associated with the tumor invasion process and that its actions can be mediated by the receptor for formylated peptides (FPR). Therefore, we evaluated the expression of ANXA1, the receptor FPR2 and matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) in brain astrocytomas. Detection of proteins was performed in sections of diffuse astrocytomas (grade II), anaplastic astrocytomas (grade III) and glioblastomas (GBM, grade IV) and quantifications were made by densitometry. Our analyses showed increased expression of ANXA1 in astrocytomas of all grades, but especially in GBM. The expression of FPR2 is similar to that found for ANXA1, being higher in GBM. Immunostaining for MMPs is also stronger as the degree of malignancy increases, especially with respect to MMP-9. The positive correlation between ANXA1/FPR2 and ANXA1/MMP-9 was observed in all tumors studied. The data indicate the possible action of ANXA1 and FPR2 on the development and progression of astrocytomas, related to increased expression of MMP-9. Thereby, ANXA1 and FPR2 are involved in the biology and malignancy of diffuse astrocytic tumors.

1. Introduction

Gliomas are the most common primary malignancies of the central nervous system (CNS) [1]. Among them, astrocytomas represent the majority and can be classified on the basis of combined phenotypic and genotypic classification according to the World Health Organization (WHO) [2]. The new classification combines histopathological and molecular features and includes the analysis of isocitrate dehydrogenase enzyme (IDH)-1 and IDH-2 mutations and 1p19q codeletion status. The CNS tumors WHO grade II diffuse astrocytomas, WHO grade III anaplastic astrocytomas and WHO grade IV glioblastomas (GBM) are now each divided into IDH-mutant, IDH-wildtype and NOS (i.e., not otherwise specified) categories [2,3]. Grades I and II diffuse astrocytic tumors are slow growing tumors and less aggressive, whereas the grade III and GBM are malignant gliomas characterized by high rate of proliferation and metastatic potential [1,3,4].

Invasion of glioma cells requires a complex series of events between the host and the tumor, involving the migration of tumor cells and the disintegration of the tumor matrix. Investigations have correlated the expression of vascular endothelial growth factor (VEGF) and proteolytic enzymes such as matrix metalloproteinases (MMP)-2 and MMP-9 with migration and human GBM cell invasion, indicating the importance of these proteins in the malignant progression [5-8].

Knowledge of the molecular mechanisms involved in the biology of astrocytomas is essential for identifying candidates for prognostic markers, new therapeutic targets and early detection strategies predictive of survival. Different investigations related the Annexin A1 protein (ANXA1) to the regulation of cell growth, cell transformation, tumor progression and metastasis [9-11].

In normal human brain, the expression of ANXA1 is limited to

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endothelial and ependymal cells and to subependymal astrocytes, but it is also upregulated in reactive astrocytes and microglia [12,13]. Studies indicated the importance of the protein in neuroinflammatory, neurovascular and metabolic disease and its potential for repairing blood-brain barrier damage in disease and aging [14]. Also, ANXA1 may control the noninflammatory phagocytosis of apoptotic neurons and promote resolution of inflammatory microglial activation [13]. The treatment with Ac2-26, a mimetic peptide of ANXA1, decreased leukocyte adhesion at 2 h after ischemia/reperfusion in a murine model of stroke [15].

In tumors, although ANXA1 expression in ependymomas decreased with the grade of malignancy, diffuse astrocytomas showed a significant increase of cytoplasmic ANXA1 [12]. The authors pointed that ANXA1 upregulation in astrocytomas may contribute to tumor progression and its expression profile is similar to its substrate, the epidermic growth factor receptor (EGFR). Other investigation showed that the overexpression of Forkhead box M1 (FoxM1) up-regulated ANXA11 expression in glioma cells. Then, the enhanced proliferation, migration, and angiogenesis promoted by FoxM1 in vitro and in vivo occurred in an ANXA1-dependent manner [16].

Another important aspect in the biology of ANXA1 has been the study of its functional and molecular relationships with the FPR receptor family [17]. The members of the human FPR family include FPR1, FPR2 (formerly known as FPR like1 or FPRL1) and FPR3 [17]. The receptors FPR1 and FPR2 share 69% of similarity [18].

ANXA1 protein can control tumor growth and invasiveness by means of paracrine mechanisms mediated by FPRs [10,19,20]. In prostate cancer cells ANXA1 induced invasiveness acting by FPR1 and FPR2, possibly through the expression of MMP2 and MMP-9, focal adhesion kinase (FAK), E-cadherin and vimentin [19]. ANXA1 is also involved in the melanoma invasion process by promoting MMP-2 expression through FPR1 and FPR2 receptors [20]. Another study showed that treatment of Hep-2 tumor cells (derived from laryngeal epidermal carcinoma) with the Ac2-26 peptide reduced the expression of the *MMP-2* gene and increased that of the *MMP-9* gene, relative to the control cells, through the interaction with the FPR2 receptor [10].

A possible association among FPR2, mitogen-activated protein kinases (MAPKs), astrocytic activation and the inflammatory response was identified in human U87 astrocytoma cells [22]. Other research pointed out that FPR2 requires lipid raft and extracellular signal-regulated protein kinase to activate inhibitor-kappa B kinase in human U87 astrocytoma cells [23]. Furthermore, FPR2 exerted considerable control over vascular inflammatory responses during cerebral ischemia and reperfusion, which provides evidence that FPR2 may be beneficial for patients who suffered from stroke [15].

In brain tumors, the expression of FPR2 is still poorly studied, but investigations indicate that the expression of FPR1 is responsible for increased motility and invasiveness of GBM cells [24]. Investigations pointed out that FPR1 expressed by human GBM cells plays a pivotal role in tumor cell growth, invasion and production of angiogenic factors [25]. FPR1 has been shown to be capable of transactivating the EGFR in GBM cells, so that both receptors cooperate to promote the malignant behavior of GBM cells [25,26].

With these considerations, we sought in this work to correlate the expression of ANXA1 with FPR2 and MMP-2 and MMP-9 as a way to contribute to better understanding the role of these proteins in diffuse astrocytic brain tumors.

2. Materials and methods

2.1. Biopsies

The analyses were performed on biopsies of diffuse astrocytic tumors WHO grade II diffuse astrocytomas, NOS (n = 12), WHO grade III anaplastic astrocytomas (n = 17), NOS and WHO grade IV glioblastomas (GBM), NOS (n = 20). The tumor material was obtained from the files (years 2000 to 2015) of the Service of Pathology, Medical School of Padre Albino University Center, Catanduva, São Paulo, Brazil, after approval of the Committee of Ethics in Research (Protocol 258.431).

The study included only biopsies of primary tumors and from patients with no preoperative chemotherapy or radiotherapy The ages of patients varied from 41 to 77 years, being mostly men over 55,2 years. Tumors were removed from the frontal (55%), temporal (30%) and parietal lobes (15%) by subtotal and total resections.

2.2. Immunohistochemical and densitometric studies

Paraffin included biopsies were used to obtain 5 μm sections that were stained by Hematoxylin-Eosin (HE) for histopathological study. Expressions of ANXA1, FPR2, MMP-2 and MMP-9 were verified in the sections of the selected biopsies, using the following rabbit polyclonal primary antibodies: anti-ANXA1 (1:3000) (Zymed Laboratories, Cambridge, UK); anti-FPR2 (1:2000) anti-MMP-2 and anti-MMP-9 (1:400) (Abcam, Cambridge, MA, USA).

The sections were prepared on silanized slides, deparaffinized and rehydrated. After the antigenic recovery (citrate buffer pH 6.0 at 96 °C for 20 min) and endogenous peroxidase blockade they were incubated in a humid chamber at 4 °C with the primary antibodies diluted in 1% BSA. Then the sections were incubated with biotinylated secondary antibody (kit Histostain, Invitrogen), developed with DAB substrate (kit DAB, Invitrogen) and counterstained with hematoxylin. The negative control of reaction was performed by omission of the primary antibody.

Analysis of the expression of the proteins was performed in all tumors slides by optical densitometry (arbitrary units 0 to 255) using the Leica Image Analysis program. From the images obtained by the 40× objective in the DM50 microscope (Leica, Germany) 20 random points were analyzed in 3 different immunostained regions of each tumor to obtain a mean related to the intensity of the immunoreactivity [27,28].

2.3. Statistical analyses

The results were previously submitted to descriptive analysis and determination of normality, and the means were compared by Analysis of Variance (ANOVA), followed by the Bonferroni test. ANXA1 was correlated to the other proteins by the Pearson test. All values were expressed as mean ± S.E.M., and P values < 0.05 were considered statistically significant. The correlation results were indicated as positive or negative.

3. Results

3.1. Overexpression of ANXA1, FPR2, MMP-2 and MMP-9 in GBM

Immunohistochemical analyses showed expression of ANXA1, FPR2 receptor, MMP-2 and MMP-9 in all clinical conditions studied (Fig. 1). The specificity of the reactions was confirmed by the respective reaction controls (Fig. 1D, H, L and P). The densitometric observations revealed increased expression of ANXA1 in grade III anaplastic astrocytomas, NOS ($P < 0.01$) compared to grade II diffuse astrocytomas, NOS and overexpression in GBM, NOS in relation to grades II ($P < 0.001$) and III ($P < 0.05$) (Fig. 1A, B, C and Q). Interestingly, the expression of FPR2 is similar to that found for ANXA1, being greater in GBM (Fig. 1E, F, G and R). Immunoreactivity for MMPs (Fig. 1I–P, S and T) is also stronger as the degree of malignancy increases, especially with respect to MMP-9 (Fig. 1M, N, O, and S).

3.2. ANXA1, FPR2 and MMP-9 are positively correlated in astrocytomas

Correlation analyses indicated that in all tumors studied, ANXA1 and FPR2 and ANXA1 and MMP-9 are positively correlated (Table 1). Differently, no positive or negative correlation was observed between