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Neuroscience Letters 394 (2006) 57–62

Letters

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Tubulin nitration in human gliomas

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Received 8 July 2005; received in revised form 30 September 2005; accepted 3 October 2005

Abstract

Immunohistochemical and biochemical investigations showed that significant protein nitration occurs in human gliomas, especially in grade IV glioblastomas at the level of astrocytes and oligodendrocytes and neurones. Enhanced alpha-tubulin immunoreactivity was co-present in the same elements in the glioblastomas. Proteomic methodologies were employed to identify a nitrated protein band at 55 kDa as alpha-tubulin. Peptide mass fingerprinting procedures demonstrated that tubulin is nitrated at Tyr224 in grade IV tumour samples but is unmodified in grade I samples and in non-cancerous brain tissue. These results provide the first characterisation of endogenously nitrated tubulin from human tumour samples.

Keywords: Gliomas; Nitrotyrosine; Immunohistochemistry; Proteomics; Tubulin

Malignant neoplasms of the central nervous system express abnormally high levels of nitric oxide synthase (NOS) [5] suggesting that nitric oxide (NO) and related nitrogen species may be associated with pathophysiological processes important to these tumours. NO can diffuse from the site of production and can interact with superoxide to generate peroxynitrite, a highly reactive species that may be responsible for nitration of tyrosine residues in proteins to form 3-nitrotyrosine [13]. Tyrosine nitration may lead to profound structural alterations with losses in specific biological functions and enhanced proteolytic degradation by the proteasome [13,18]. However, moderate tyrosine nitration also occurs in healthy tissues without causing apparent dysfunctions in target proteins [2]. Protein nitration was recently suggested to be involved in signal transduction [13], cell differentiation [3] and normal embryo development [10].

Increased levels of nitrated proteins have been detected in several diseases [11,20] and in some forms of cancer [7,12]. Little is known about the target proteins for endogenous nitration during brain tumour progression, though elevated levels of nitrated proteins, as well as increased expression of inducible NOS constitute an established correlate of human gliomas [5,6,9]. A candidate target for nitration is represented by the cytoskeletal protein tubulin. Tubulin nitration is putatively involved in protein turnover through the tyrosination/detyrosination cycle [14]. When free 3-nitrotyrosine is present in the cell, it is irreversibly incorporated into alpha-tubulin preventing its detyrosination which is essential in cellular growth, differentiation and motility [4,8]. Nitrotyrosination of alpha-tubulin induces alterations in cell morphology and changes in microtubule organisation [8], though recent data showed that 3-nitrotyrosine incorporation into alpha-tubulin is reversible and not detrimental to cells [1]. In this regard, recovery mechanisms restoring basal levels of functionally active alpha-tubulin appear to be operative in some invertebrate systems [17].

In this paper, we used biochemical, immunohistochemical and proteomic approaches to characterise endogenous protein nitration in human gliomas. We demonstrated that tubulin is a target of nitration in these tumours with modification essentially occurring at a specific tyrosine residue different from that pre-

Abbreviations: ES/MS, electrospray mass spectrometry; ES/MS/MS, electrospray tandem mass spectrometry; HPLC, high-performance liquid chromatography; LC/MS/MS, liquid chromatography–electrospray tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionisation/time of flight; NOS, nitric oxide synthase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid

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doi:10.1016/j.neulet.2005.10.011

viously reported under physiologically relevant conditions in PC12 cells [19].

Brain tumour specimens were obtained from patients operated on at the Neurosurgery Department, Second University of Naples. All patients or legal guardians gave informed consent for this study (according to the Declaration of Helsinki and the Institutional Review Board of the Second University of Naples). They were given detailed information about the aim of the research, experimental procedures and methodologies. Surgical decision-making was independent of the present study. Tumour type and grade were determined by histological assessment according to the World Health Organisation criteria for the grading of brain tumours. The grading system is grades I–IV. Grade I tumours are the least malignant and grow only very slowly, whereas grade IV tumours are more malignant and grow faster.

Patient 1: 56-year-old male, left fronto-temporal craniotomy. A rosaceous lesion infiltrating the temporal tip was excised. Histological examination showed low-to-mild proliferation of pseudo-spongioblastic glial fibrillar acidic protein-positive elements with Rosenthal fibres. Diagnosis: grade I pilocytic astrocytoma.

Patient 2: 45-year-old male, who underwent left frontal craniotomy to remove a frontal lesion (3 cm diameter) localised by echo guidance at a depth of 2 cm. Histological examination showed high proliferation of cells with monotonous round nuclei with eccentric rim of eosinophilic cytoplasm. Diagnosis: grade II oligodendroglioma.

Patient 3: 53-year-old male, operated for right frontal glioblastoma 2 years earlier, who underwent right frontal craniotomy for a recurrent tumour. An intraoperative ecography localised a neoplasm at 1 cm depth from the smooth frontal cerebral circumvolutions. Histological examination showed proliferation of anaplastic elements with high mitotic activity and neovascularisation as well as areas of necrosis. Diagnosis: grade IV glioblastoma.

Patient 4: 64-year-old male, left frontoparietal craniotomy. The lesion (3 cm maximal diameter) was localised with echo guidance at a depth of 2 cm. Histological examination showed high proliferation of small lymphocyte-like elements with high mitotic activity and frequent necrotic areas and pseudorosette. Diagnosis: grade IV glioblastoma.

Patient 5: 79-year-old female, parieto-occipital craniotomy. A rosaceous cortical–sub-cortical neoplasm was completely removed. Histological examination showed high proliferation of glial anaplastic and plurinucleate elements with proliferation of vessels, and haemorrhagic areas. Diagnosis: grade IV glioblastoma.

Pathological specimens obtained from neurosurgery were either frozen immediately at −80 °C and analysed within 1 day, or prepared for immunohistochemistry. Trypsin, dithiothreitol, iodoacetamide and α-cyano-4-hydroxycinnamic acid were purchased from Sigma. High-performance liquid chromatography (HPLC) grade trifluoroacetic acid (TFA) was from Carlo Erba. All other reagents and solvents were from Baker and were of the highest purity available.

Brain samples were homogenised in 25 mM Tris buffer (pH 7.4) containing 100 mM NaCl, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 18,000 × *g* for 20 min and the extracts were subjected to western blotting for nitrated protein analysis. Protein concentration was determined using a Bio-Rad protein assay reagent (Bio-Rad, Milan, Italy) with bovine serum albumin as a standard.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as reported [17] using an acrylamide concentration of 7.5%. Rabbit polyclonal antibody against 3-nitrotyrosine (1:1000) (Chemicon) was used.

For immunohistochemistry, tissues were treated as previously described [17]. The primary antibodies were rabbit polyclonal antibody against 3-nitrotyrosine (1:100) (Chemicon) and mouse monoclonal anti-alpha-tubulin (1: 5000) (Sigma).

Coomassie blue-stained protein bands were excised from SDS gels and in situ digested as described previously [15].

Matrix-assisted laser desorption ionisation/time of flight (MALDI-TOF) mass spectra were recorded using an Applied Biosystem Voyager DE-PRO instrument. A mixture of analyte and matrix solution (alfa-cyano-hydroxycinnamic acid 10 mg/ml in 66% acetonitrile, 0.1% TFA, in MilliQ water) was applied to the metallic sample plate and dried down at room temperature. Mass calibration was performed using external peptide standards. Raw data were analysed using the computer software provided by the manufacturer and reported as monoisotopic masses. Peptide masses of each digested protein were used to search for protein databases using three different mass fingerprinting softwares available on the net: MsFit from Protein Prospector, Mascot from Matrix Science and ProFound from Prowl.

Tryptic peptide mixtures obtained from in situ digestions were also analysed by “on-line” Liquid Chromatography–Electrospray Tandem Mass Spectrometry (LC/MS/MS) using an LCQ ion trap instrument (Finnigan Corp., San José, CA, USA). Proteolytic digests were fractionated on a HP 1100 HPLC apparatus (Hewlett-Packard, Palo Alto, CA, USA) using a narrowbore Phenomenex Jupiter C18 column (250 mm × 2.1 mm, 300 Å) (Torrance, CA, USA) with 0.05% TFA, 5% formic acid in MilliQ water (solvent A) and 0.05% TFA, 5% formic acid in acetonitrile (solvent B) by means of a linear gradient from 5 to 70% solvent B in 60 min at a flow rate of 0.2 ml/min. The effluent was directly inserted into the ion source through the electrospray probe and both electrospray mass spectrometry (ES/MS) and electrospray tandem mass spectrometry (ES/MS/MS) spectra were acquired throughout the entire analysis by using the software provided with the instrument.

Protein nitration was determined in five brain tumour surgical specimens, including astrocytomas, oligodendrogliomas and highly malignant glioblastomas. Elevated tyrosine nitration was observed in all tumours as determined by nitrotyrosine immunoreactivity. However, significant differences were observed in individual samples as reported in Fig. 1. In astrocytoma (grade I), immunoreactivity to nitrotyrosine was confined to few glial cells of different size. Some neurones showed positive immunoreaction, and no response was observed in the stroma (Fig. 1A). In oligodendroglioma (grade II) glial cells exhibited cytoplasmic immunoreactivity to nitrotyrosine. A

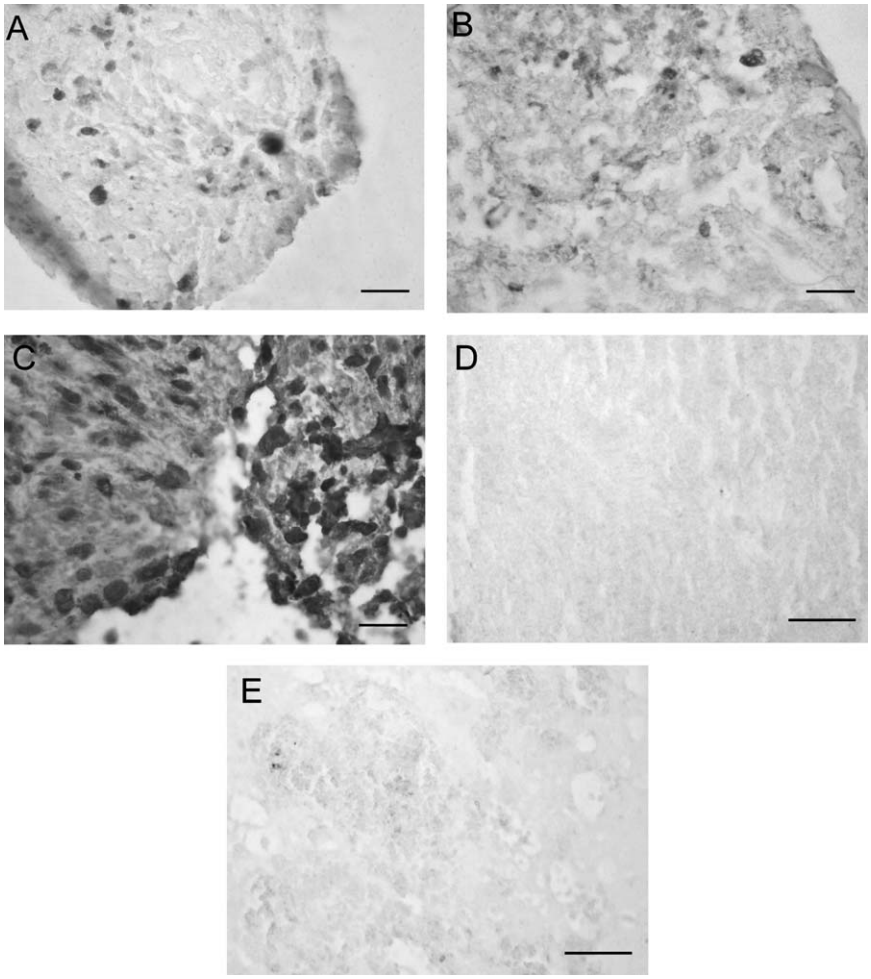

Fig. 1. 3-Nitrotyrosine immunohistochemical staining of human gliomas. (A) Astrocytoma (grade I). Immunoreactivity is confined to spherical glial cells. Stain was absent in the stroma. (B) Oligodendroglioma (grade II). Immunoreactivity is present in glial cells and a weak stain is present in the stroma. (C) Representative glioblastoma (grade IV; sample 5). Immunoreactivity is present in many glial and neuronal elements. Note the very intense immunostaining in the stroma. (D) Pre-absorption of anti-3-nitrotyrosine with 3-nitrotyrosine as control completely blocked immunostaining. (E) Sections of areas of minimally invaded brain from the same pathological sections of the glioma patients stained with antibodies to 3-nitrotyrosine. Little or no nitration was observed. Scale bars = 100 μm.

weak and diffuse immunoreactivity was observed in the stroma (Fig. 1B). In all samples of malignant glioblastomas (grade IV) which are characterised by rapid growth, vascular proliferation and oedema, and express relatively high levels of NOS I and NOS II [5] the patterns of nitrotyrosine immunoreactivity were similar. Strong nitrotyrosine immunopositivity was present in many glial as well as neuronal elements (Fig. 1C). In some cases immunopositive cells seemed to be plurinucleated. Along the necrosis areas, immunoreactive elements were organised as clusters. Strong immunoreactivity was also observed in the stroma. Pre-incubation of the nitrotyrosine antibody with 3-nitrotyrosine as control completely blocked immunostaining from a serial section of the same tumour (Fig. 1D). Little or no nitration was observed in areas of minimally invaded brain from the same pathological sections of the glioma patients (Fig. 1E).

Investigation on the presence of alpha-tubulin in the same samples assayed for nitrotyrosine immunoreactivity was performed using specific anti-tubulin antibodies (Fig. 2). Tubulin immunoreactivity was normally observed in both neuronal and glial elements. However, immunohistochemical analyses revealed that tubulin location within the different classes of elements changes as the degree of malignancy increases, indicating a non-uniform expression of the antigen. In fact, in both astrocytoma (grade I, Fig. 2A) and oligodendroglioma (grade II, Fig. 2B), as well as in not invaded areas (Fig. 2E), immunopositivity to the alpha-tubulin was identified in neuronal elements, especially fibres and nerve endings. In the oligodendroglioma, however, some glial cells displayed an intense immunostaining (Fig. 2B). In the malignant glioblastoma (grade IV), even though immunoreactivity is still present in the neuronal elements, tubulin was strongly immunodetected in the glial elements (Fig. 2C). Controls performed by omitting primary antibody revealed no tubulin immunoreactivity (Fig. 2D).

Identification of nitrated proteins in tumour samples was accomplished by proteomic approaches. Protein extracts from the five brain tumour samples were fractionated by SDS–PAGE (Fig. 3). All extracts exhibited well-detectable nitrated protein bands at 33 (a), 40 (b) and 55 kDa (e), four showed an additional band at 45 kDa (c) and one had a distinct band at 50 kDa (d). The Coomassie-stained bands corresponding to the immunore-

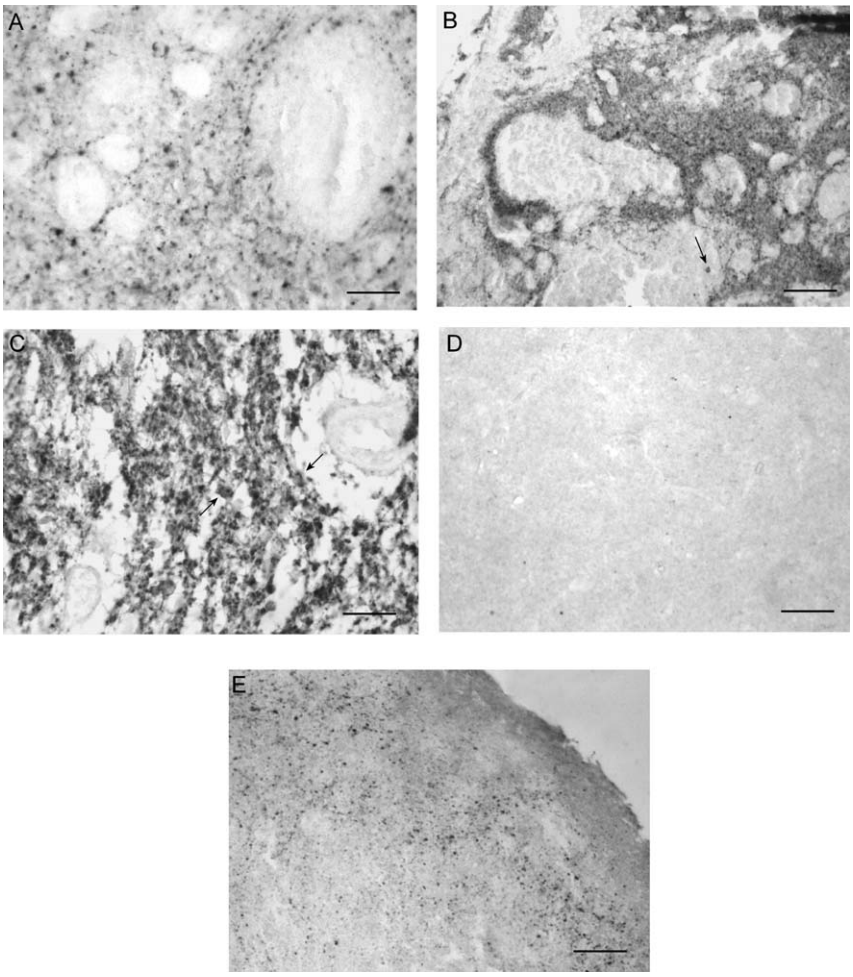

Fig. 2. Alpha-tubulin immunoreactivity in human gliomas. (A) Astrocytoma (grade I). Immunoreactivity is present in fibres and nerve endings. (B) Oligodendroglioma (grade II). Immunoreactivity is mainly present in fibres, but some glial cells show immunoreactivity (arrow). (C) Glioblastoma (grade IV; sample 5). Alpha-tubulin strong immunopositivity is widely distributed to many glial (arrows) and neuronal elements. (D) Omission of anti-alpha-tubulin as control completely blocked immunostaining. (E) Sections of areas of minimally invaded brain from the same pathological sections of the glioma patients stained with antibodies to alpha-tubulin. Immunostaining is present in fibres. Scale bars = 70 μm in (A and C); 150 μm in (B, D and E).

active proteins were excised from the gels, reduced, alkylated and in situ digested with trypsin. Fig. 4 shows the partial MALDI mass spectrum of a peptide mixture from the tryptic digest of the 55 kDa protein from sample 4, taken as representative sample. Mass signals were used to search for a non-redundant sequence database using three different softwares available on the net, taking advantage of the specificity of trypsin and the taxonomic category of the sample. The set of mass values was compared to the theoretically predicted peptides from the proteins in the explored database. A set of 17 mass values matched within human tubulin alpha 6 sequence with a mass accuracy better than 80 ppm.

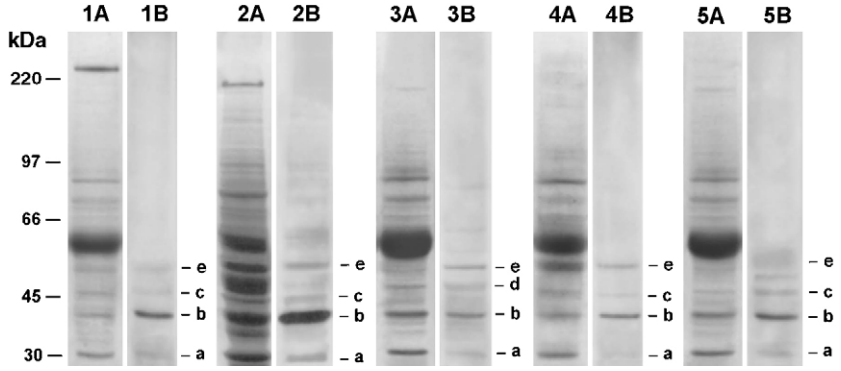

Fig. 3. SDS electrophoresis of protein extracts from brain tumour samples. Lanes 1A–5A: Coomassie-stained gels. Lanes 1B–5B: Western Blot. Lane numbers correspond to patient identification numbers.

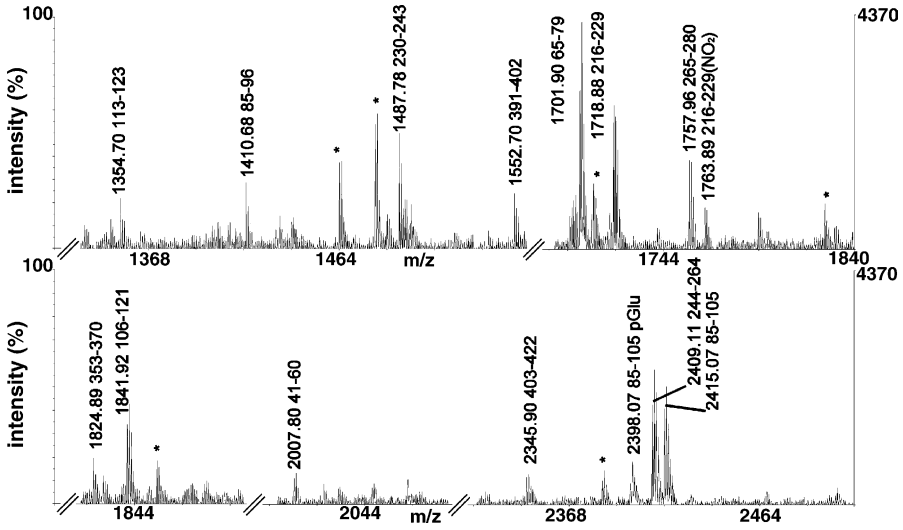

Fig. 4. Partial MALDI mass spectrum of the peptide digest from the band at 55 kDa. Mass signals were assigned to the corresponding peptides within tubulin alpha 6 sequence on the basis of their mass values. Nitrated 216–229 peptide is indicated with (NO_2). Trypsin autoproteolysis peaks are marked with an asterisk (*).

All the other putative candidates received a very low probability score, thus leading to the identification of tubulin as the protein component. The mass spectral analysis led to a sequence coverage of the tubulin primary structure greater than 46%. Moreover, investigation of the MALDI spectrum led to the identification of the nitration site. Two related signals at *m*/*z* 1763. 9 and 1718.9, respectively, were detected in the spectrum that exhibited a mass difference of 45 Da, corresponding to a nitro group. The peak *m*/*z* 1718.9 was assigned to the fragment 216–229 within tubulin sequence whereas the satellite signal at *m*/*z* 1763.9 corresponded to the peptide carrying a 3-nitro tyrosine residue. Since this fragment contains a single Tyr residue at position 224, the modification site was unambiguously determined.

Although a high percentage of the protein sequence could be mapped by MALDI mass fingerprinting, some sequence portions escaped identification. The peptide mixture was then submitted to LC/MS/MS analysis. The peptide sequences obtained from the interpretation of tandem mass spectra were used to confirm MALDI identification and led to an increase in the sequence coverage which raised to 53%. Several tyrosine containing peptides were identified by the mass spectrometric procedures, thus leading to the identification of 10 Tyr residues that resulted to be unmodified (Tyr 83, Tyr 103, Tyr 108, Tyr 262, Tyr 272, Tyr 312, Tyr 319, Tyr 357, Tyr 393, Tyr 408). However, a few tyrosine residues escaped identification and their modification state could not be monitored.

The presence of tubulin in the protein extracts from the remaining four brain tumours (samples 1, 2, 3 and 5) was assessed using the same procedure. However, different results on the occurrence of 3-nitrotyrosine in tubulin in the different tumour grades were obtained. As an example, Fig. 5 shows the partial MALDI spectrum of the peptide mixtures from the tryptic digest of the tubulin band in malignant glioblastoma (grade IV; sample 4, panel a) and in astrocytoma (grade I; sample 1, panel b). The mass signal corresponding to the nitrated form of the peptide 216–229 was clearly detected at *m*/*z* 1763.9 in the glioblastoma sample whereas this peak was completely absent in the low-grade tumour sample. The remaining portions of the spectra of the two samples were almost identical. Similarly, the mass signals corresponding to the nitropeptide 216–229 were detected in the other glioblastoma samples. No detectable tyrosine nitration was observed in the peptide 216–229 in non-cancerous brain tissue (data not shown).

In this study we reported immunohistochemical evidence indicating elevated protein nitration and tubulin expression in

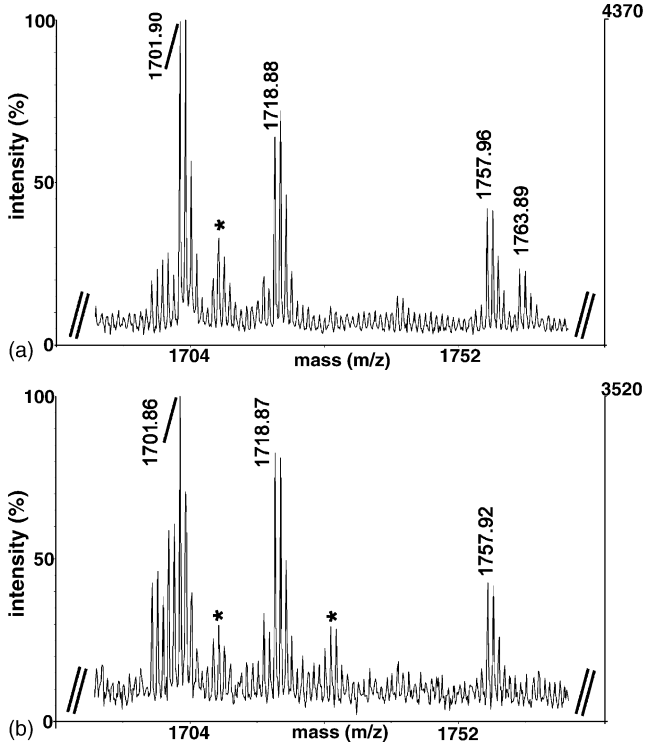

Fig. 5. Partial MALDI mass spectrum of the mixtures from the tryptic digest of the tubulin band in malignant glioblastoma (grade IV; sample 4, panel a) and in astrocytoma (grade I; sample 1, panel b).

human gliomas compared to non-cancerous brain tissues, with an apparently higher immunochemical response in glioblastoma (grade IV) samples. Tubulin immunoreactivity becomes maximal in the glial elements as the neoplasm progresses, mirroring the results of nitrotyrosine immunodetection. Finally, both antigens are extremely immunoreactive in glial cells in advanced grade tumours.

Proteomic investigation of protein extracts from grade IV tumour tissues provided direct evidences that tubulin is nitrated under severely pathological conditions. In particular, peptide mass fingerprinting procedures allowed us to identify Tyr224 as a specific nitration site of alpha-tubulin in grade IV human glioma samples. On the contrary, this residue was found to be unmodified in lower grade tumours, e.g. astrocytoma (grade I) or in control tissues. To the best of our knowledge, this is the first identification of an in vivo site of endogenous nitration of alpha-tubulin in human tumour tissues. Mass spectral analyses led to the identification of 10 further Tyr residues that were found unmodified.

Alpha-tubulin contains 18 or 19 tyrosine residues depending on the presence of the C-terminal tyrosine which can be lost following specific proteolytic processing events [21]. These residues are not equally susceptible to nitration, and several structural factors responsible for selective post-translational modification have been proposed. A mass spectrometric characterisation of nitrated alpha-tubulin in PC12 cells undergoing neuronal differentiation following exposure to nerve growth factor has revealed significant nitration at the 161 and 357 tyrosine residues [19]. Data reported in this paper demonstrated that at least one of these residues, Tyr357, is unmodified in tumour tissues. These results might suggest that different tyrosine residues are susceptible to nitration under different biological conditions, i.e., in physiological or pathological conditions, although the limitations inherent to the MS methodology must be duly kept into account. Tyr 224 belongs to the inner, drug-binding domain of alpha-tubulin [16] and is evidently exposed to solvent and nitrating agents, as reported for the bovine protein [19]. Whether and how this modification affects interaction with other tubulin subunits or microtubule associated proteins and the relevance to altered neuronal cell growth remains to be determined and represents an important goal for future studies on protein nitration in human glioma.

Acknowledgements

This work was supported in part by grants from Italian MIUR (PRIN 2003 to M.d'I. and FIRB 2003 to PP) and CNR (to M.d'I.).

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