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Expression of hypoxia-inducible factor-1α (HIF-1α) in pituitary tumours

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Summary. The present study was performed to investigate HIF-1α (hypoxia-inducible factor-1α) expression in a large number of immunohistochemically and ultrastructurally characterized surgically removed pituitary tumours. The potential relation of HIF-1α with outcome variables as well as the presence of HIF-1α expression in the tumours treated with dopamine agonists and octreotide, a long-acting somatostatin analogue was also investigated. HIF-1α immunoreactivity was confined to the nucleoplasm whereas the nucleoli were unconspicuous. The distribution of HIF-1α was evident in the tumours whereas normal adenohypophysial cells showed no HIF-1α staining. HIF-1α expression was detected not only in the tumour cells but also in endothelial cells lining the blood vessels within the tumour. ACTH producing adenomas showed the lowest level of HIF-1α expression whereas pituitary carcinomas and GH producing adenomas had the highest counts. The statistical study demonstrated no significant correlation between HIF-1α expression, patient age, gender, tumour, size, invasiveness, cell proliferation rate and vascularity. These results suggest that the behaviour of pituitary tumours does not primarily depend of HIF-1α expression. Our study demonstrated an increase HIF-1α expression in bromocriptine treated PRL producing pituitary adenomas compared with untreated tumours but no increase in octreotide treated tumours.

Key words: Angiogenesis, Pituitary tumours, Hypoxia, MIB-1, Immunohistochemistry, Hypoxia-inducible factor-1α

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Introduction

Hypoxia, disruption of oxygen homeostasis caused by low oxygen levels, is crucial in the development and progression of a large number of disorders such as coronary heart disease, stroke, pulmonary emphysema, and tumours (Bunn and Poyton, 1996; Semenza, 2001a). In various tumours, diminished free oxygen availability results when growth exceeds vascular supply. Under such conditions, changes in cellular oxygen concentration redirect cellular biosynthetic pathways to promote adaptation and enable survival. It was recently reported that a transcription factor called hypoxia-inducible factor 1 (HIF-1), plays a crucial role in the up-regulation of genes involved in hypoxia adaptive mechanisms. These genes include those encoding erythropoietin, glucose transporters, glycolytic enzymes, vascular endothelial growth factor (VEGF), transferrin, hemoxygenase, and inducible nitric oxide synthase (Semenza et al., 1996; Semenza, 1999, 2001b; Rivard et al., 2000).

HIF-1 is a heterodimeric complex composed of two basic helix-loop-helix (bH-L-H) PAS subunits termed HIF-1α and HIF-1ß (Wang and Semenza, 1995; Wang et al., 1995). Whereas HIF-1ß is the common subunit of multiple bH-L-H proteins and is constitutively expressed, HIF-1α is the unique O_2-regulated subunit that determines HIF-1 activity. HIF-1α is a short-lived protein. Its levels are tightly regulated by oxygen concentration via the ubiquitin-proteosome. In normoxia, HIF-1α is maintained at low and often undetectable levels. In hypoxia, however, the protein is translocated to the nucleus rapidly and strongly increasing HIF-1α by reducing its proteosome-dependent degradation (Iyer et al., 1998). Although previous studies have demonstrated that HIF-1α is overexpressed in the majority of human cancers (Zhong et al., 1999; Zagzag et al., 2000; Giatromanolaki et al., 2001), such as those of breast, colon, lung, and prostate, the role of HIF-1α in tumour progression is still not

clear. It has been reported that HIF-1$\alpha^{-/-}$ tumours show an accelerated growth compared to HIF-1$\alpha^{+/+}$ tumours, thus suggesting that loss of HIF-1α in tumour cells renders them less dependent upon vascular supply (Carmeliet et al., 1998). Pituitary adenomas exhibit low-level angiogenesis. This is reflected in diminished microvessel density and decreased VEGF expression compared to those of nontumourous anterior pituitary (Jugenburg et al., 1995; Lloyd et al., 1999; Vidal et al., 1999, 2000; Turner et al., 2000a). Although it has been suggested that lack of angiogenesis underlies the slow pace of pituitary tumour growth, prior studies were unable to demonstrate a correlation between tumour vascularity and cell proliferation (Turner et al., 2000b,c; Vidal et al., 2001).

Since HIF-1α might play an important role in pituitary tumour angiogenesis and growth, this study was undertaken to investigate its expression in an immunohistochemically and ultrastructurally characterized large series of pituitary tumours. Our aim was to correlate the degree of HIF-1α expression with patient age, gender, tumour type, size, cell proliferation, vascularity, invasiveness, and metastatic potential. HIF-1α expression was also investigated in adenomas treated with bromocriptine, dopamine agonist and octreotide, a long-acting somatostatin analogue.

Materials and methods

Patients

A series of 155 pituitary tumours, including adenomas and carcinomas, was selected from the Mayo Clinic tissue registry and from the consultation files of three of the authors (BWS, KK, EH). The material included tissues removed by transsphenoidal surgery from 70 men (mean age 52.9, range 11-81) and 85 women (mean age 45.7, range 17-80). Based on histology, immunohistochemistry, and selective transmission electron microscopy, each tumour was evaluated and placed into one of the following six categories: GH-producing adenomas (10 cases); PRL-producing adenomas (37 cases); ACTH-producing adenomas (10 cases with Cushing's disease); TSH-producing adenomas (10 cases) and clinically non-functioning adenomas (including 8 ACTH silent subtype 1, 13 silent subtype 3, 10 female and 10 male gonadotroph adenomas, 10 non-oncocytic and 10 oncocytic null cell adenomas). Also studied were 10 bromocriptine-treated PRL-producing adenomas and 10 octreotide-treated GH-producing adenomas. In addition, 7 pituitary carcinomas (4 PRL-producing and 3 ACTH-producing tumours) were examined. Tumour size, invasiveness, proliferative activity, and vascularity were evaluated in each case. Size and invasiveness were assessed on the basis of preoperative magnetic resonance imaging (MRI) scans and operative findings. Tumours were divided into micro- or macroadenomas, defined as tumours <1 cm and >1 cm, in their diameter.

Morphology

All specimens were promptly fixed in 10% buffered formalin, routinely processed, paraffin embedded and cut at 5 μm. Each tumour was characterized by histology [hematoxylin and eosin (H+E), periodic acid Schiff (PAS), Gordon-Sweet silver method] and by immunohistochemistry for pituitary hormones using the labeled streptavidin-biotin peroxidase complex method. Antisera were directed against the complete spectrum of pituitary hormones, including growth hormone (GH), prolactin (PRL), adrenocorticotropic hormone (ACTH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyrotropic hormone (TSH), and the alpha subunit of glycoprotein hormones. The sources, dilutions, and clonality of these antibodies, as well as control methods have been previously described (Kovacs et al., 1989, 1991). Many tumours were also glutaraldehyde fixed, routinely processed, Epon-embedded, and ultrastructurally studied on a Philips 410LS transmission electron microscope.

Immunostaining was also performed to analyze Ki-67 expression and tumour vascularity using the monoclonal antibodies MIB-1 and CD-34, respectively. Details of immunohistochemistry, including duration of exposure and control procedures have been described in previous publications (Vidal et al., 1999, 2001). Immunohistochemical results were assessed quantitatively. MIB-1 labeling, defining the proliferative activity of the tumour, was expressed as "percent positive nuclei" in randomly selected fields (Vidal et al., 2001). A mean of 30 fields, each containing approximately 100 cells, was assessed in all cases. Cells considered positive showed unequivocal nuclear staining. CD-34 immunostained sections were examined using a computer analysis system (Microimage, Media Cybernetics, MD) to determine vascularity (Vidal et al., 1999, 2001). Measured parameters included microvessel density (the percentage of pituitary occupied by vessels) and microvessel surface density (the percentage of the vessel circumference in direct contract with pituitary tissue). In each specimen, a total of 20 randomly selected fields, corresponding to 6.7 mm^2 of pituitary tissue, were assessed. HIF-1α indices were determined immunohistochemically using the tyramide amplification technique to increase its sensitivity (Sanno et al., 2001). Staining was performed with a mouse monoclonal antibody recognizing the α isoform of HIF-1 (Novus Biologicals, Inc, Littleton, CO). Preliminary titration experiments determined the optimal working dilution to be 1:1000. After routine deparaffinization, rehydration, and blocking of endogenous peroxidase activity, sections were incubated with the antiserum, exposed to the streptavidin-biotin peroxidase complex, and incubated in biotinyl tyramide (amplification reagent) and streptavidin-peroxidase. Diaminobenzidine served as the chromogen. Sections of positive controls (mammary and colon carcinomas) were included in each batch. To confirm the specificity of the primary

antibody, control tests included: a) replacement of HIF-1α primary antibody with phosphate buffered saline, and, b) omission of streptavidin peroxidase or biotinyl tyramide.

Sections immunostained for HIF-1α were assessed using the same computer image analysis system. All quantitative evaluations were performed blindly by one of the authors (SV). For each case, the HIF-1α index was determined based on the number of positively staining nuclei divided by the total number of nuclei counted. In every case, at least 1000 cells were counted from contiguous fields. Data were tested for statistical significance using the SPSS statistical computer program (SPSS, Inc., Chicago, IL). Since assumptions for a parametric test were not valid (Kolmogorov-Sminov $p<0.05$), all data were evaluated by Kruskall-Wallis analysis of variance and the Mann-Whitney U test as a multiple comparison method. The Speerman test was used to assess the statistical significance of correlations between patient age, MIB-1 labeling indices, and tumour vascularity. Differences of $p<0.05$ were considered statistically significant.

Results

HIF-1α immunoreactivity was essentially confined to the nucleoplasm; nucleoli were not substantially labeled. Nonspecific cross-reactivity with cytoplasmic or matrix antigens was not observed (Fig. 1). The distribution of HIF-1α reactivity was restricted to tumours. Normal adenohypophysis, where present, showed no staining. HIF-1α expression was detected not only in neoplastic cells but also in endothelial cells of tumoural blood vessels (Fig. 2). HIF-1α immunopositive tumour cells were randomly distributed. Immunostaining for HIF-1α demonstrated no topographic relationship between immunopositive cells and blood vessels (Figs. 3, 4). The number of positive cells varied in the different tumour types, ranging from 0 to 32% with a mean value of 6.98% (curtails 25% = 1.80%, curtails 75% = 10.20%). Most tumours, 124 of 135 untreated pituitary tumours (93%), showed HIF-1α expression. Among the tumours, expression was strong (>10% immunopositive cells) in 32 (24%), moderate (2 to 10% immunopositive cells) in 69 (51%), and weak (0 to 2% immunopositive cells) in 23 (14%). No HIF-1α staining was seen in 11 tumours (8%). Immunonegative tumours were only detected among PRL-producing adenomas, ACTH-producing adenomas, and non-functioning adenomas.

Between the different pituitary tumour types, significant differences were noted in the percentages of nuclei-expressing HIF-1α. ACTH-producing adenomas showed the lowest level of HIF-1α expression, whereas carcinomas and GH-producing adenomas had the highest counts. Expression was significantly higher ($p<0.05$) in PRL- and in ACTH-producing pituitary carcinomas than in their corresponding adenomas. Among other tumours, PRL-producing adenomas, TSH-producing adenomas, and non-functioning adenomas showed indeterminate expression of HIF-1α (Fig. 5).

Considering all types of pituitary tumours, adenomas and carcinomas, we demonstrated no statistically significant correlation between HIF-1α expression and patient age ($r=0.07$, $p=54$) or gender ($p=0.79$). HIF-1α expression was also unrelated to pituitary tumour size ($p=0.49$), and invasiveness ($p=0.48$).

Similarly, there was no firm correlation between HIF-1α expression and MIB-1 labeling indices ($r=0.02$, $p=0.85$), microvessel density ($r=0.08$, $p=0.48$), and microvessel surface density ($r=0.01$, $p=0.94$). Nonetheless, it is of note that pituitary tumours with very low microvessel density (range 0.40-1.16%, overall mean microvessel density 2.64%) showed moderate or high HIF-1α expression. In contrast, microvessel density exceeded 2.64% in 50% of HIF-1α immunonegative tumours and in 67% those showing low-level expression.

As shown in Figure 6, HIF-1α expression was significantly increased in bromocriptine-treated PRL-producing adenomas when compared to untreated PRL-producing adenomas ($p<0.05$). No correlation was observed between HIF-1α expression, MIB-1 labeling ($r=0.20$, $p=0.60$), or microvessel density ($r=0.21$, $p=0.61$) in bromocriptine-treated and untreated PRL-producing adenomas. Lastly, a significant negative correlation was observed between HIF-1α expression and microvessel surface density ($r=0.76$, $p=0.002$).

In contrast to bromocriptine-treated PRL-producing adenomas, no significant differences were noted in HIF-1α expression between octreotide-treated GH-producing adenomas and untreated adenomas of this type ($p=0.95$). In addition, in those tumours, no significant correlation was found between HIF-1α expression, MIB-1 labeling indices ($r=0.07$, $p=0.78$), microvessel density ($r=0.09$, $p=0.76$) or microvessel surface density ($r=0.20$, $p=0.52$).

Discussion

Hypoxia is known to affect many physiological and pathological processes. For example, in postnatal life, it elicits a variety of cellular and systemic physiologic responses, including angiogenesis, erythropoiesis, and glycolysis (Semenza et al., 1996; Rivard et al., 2000; Semenza, 1999, 2001a,b). It has also been shown that oxygen availability plays a crucial role in the development and progression of malignant tumours, eg. low oxygen tension may a) cause the emergence of drug/radiation-resistant tumour cells, b) enhance mutagenesis, and c) increase metastatic potential (Young et al., 1988; Sakata et al., 1991; Kalra et al., 1993; Hockel et al., 1996a,b; Brizel et al., 1999, 2001; Vaupel et al., 2001). Collectively, these findings suggest that hypoxia may alter gene expression in neoplastic cells. Recent studies provided evidence that HIF-1α is involved in up-regulated transcription of diverse genes (Giatromanolaki et al., 2001). Talks et al. (2000) have recently reported that HIF-1α is up-regulated in many human tumours, including those of breast, colon, pancreas, prostate, bladder, ovary, liver, kidney, and

brain. In the present study, we detected increased HIF-1α expression in the majority (93%) of untreated pituitary tumours. Since HIF-1α is labile in oxygenated cells (Salceda and Caro, 1997; Berra et al., 2001) the existence of some only relatively oxygenated pituitary tumours could explain why its expression is a common, though not universal finding in pituitary tumours. In keeping with this possibility, we found poorly vascularized pituitary tumours to show high-level HIF-1α expression. Although prior studies have documented

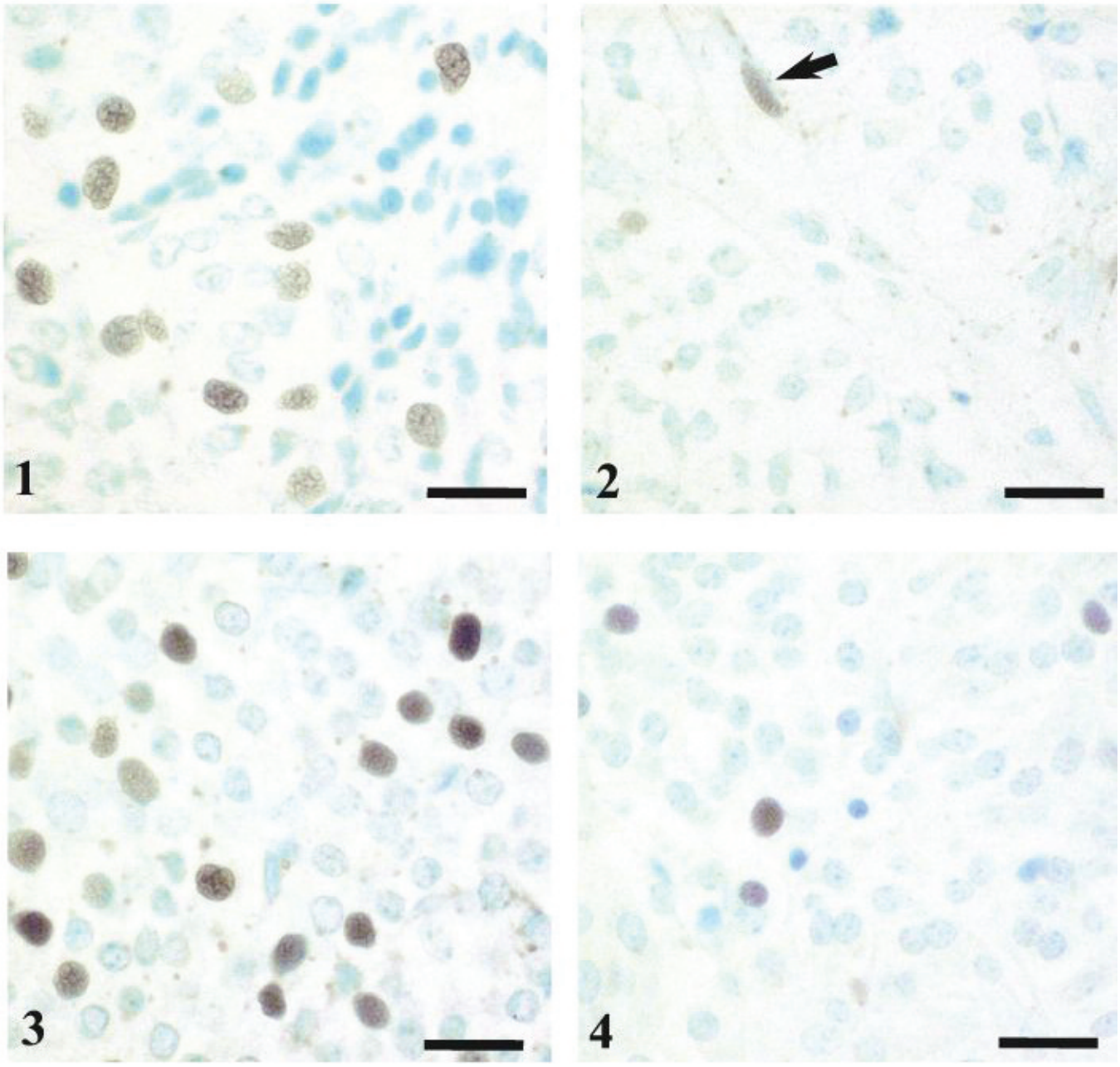

Fig. 1. Non-functioning pituitary adenoma shows intense nuclear staining for HIF-1α. Bar: 20 μm.

Fig. 2. Immunostaining shows HIF-1α immunoreactivity in the endothelial cells (arrow) of the untreated pituitary adenoma. Bar: 20 μm.

Fig. 3. Immunostaining for HIF-1α in pituitary carcinoma shows numerous positive cells. Bar; 20 μm.

Fig. 4. Immunohistochemical staining of ACTH producing adenoma for HIF-1α shows only a few positive cells. Bar: 20 μm.

a major role for HIF-1α activation in the promotion of angiogenesis in other tumour types (Maxwell et al., 1997; Carmeliet et al., 1998; Birner et al., 2001), contradictory findings have been reported with regard to pituitary tumours. Firstly, despite the well-established relationship between HIF-1α and VEGF expression in various tumour types, among pituitary tumours, the lowest HIF-1α index was found in ACTH-producing adenomas, the group known to be strongly immunoreactive for VEGF (Lloyd et al., 1999). This discrepancy suggests alternative regulatory pathways in the pituitary expression of VEGF. Previous studies have shown VEGF expression in the pituitary may play a role in the regulation of other factors, such as pituitary tumour transforming gene (Heaney and Melmed, 1999), oestrogen (Banerjee et al., 2000), and glucocorticoids (Birner et al., 2001; Lohrer et al., 2001). Secondly, our study found no significant correlation between HIF-1α expression and microvascular density. Our results are consistent with those of Giatromanolaki et al. (2001), who reported a high rate of HIF-1α expression in cases of non-small cell lung cancers with both high and low microvessel density. We also show higher levels of HIF-1α expression in pituitary carcinomas and GH-producing adenomas, the most and least vascularized of pituitary tumours, respectively (Vidal et al., 2001). Several factors might contribute to the lack of correlation between HIF-1α expression and microvessel density. One possible explanation could be related to the effect of HIF-1α upon angiogenesis. Previous studies have shown that HIF-1α is required, not for vessel formation per se, but for the regular distribution of the vascular network (Yu et al., 2001). Thus, it was suggested that disordered vasculature promotes microenvironmental hypoxia. In keeping with this hypothesis, we recently showed that, rather than

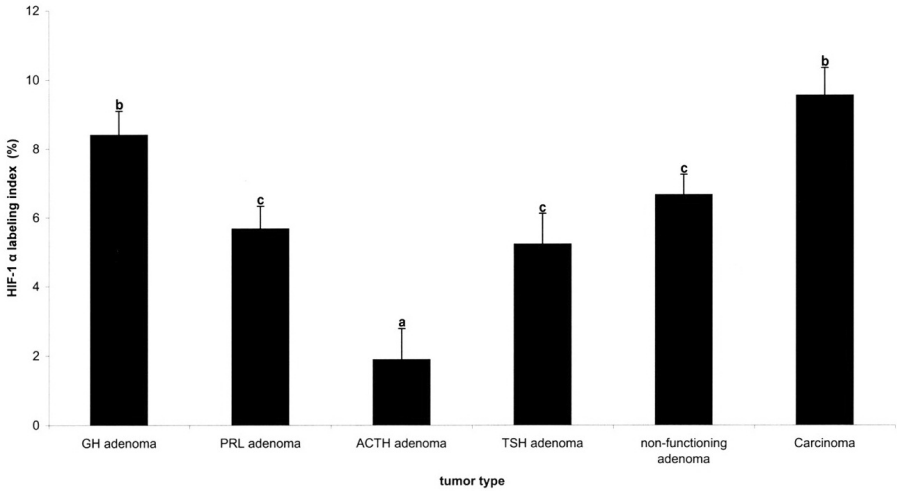

Fig. 5. Pituitary microvessel density in various pituitary tumors types. The data are expressed as percentage of HIF-1α immunopositive cells and represent the mean ± SEM. Values with no letters in common are significantly different $p<0.05$ (statistical analysis with the Kruskall-Wallis analysis of variance and the Mann-Whitney U test).

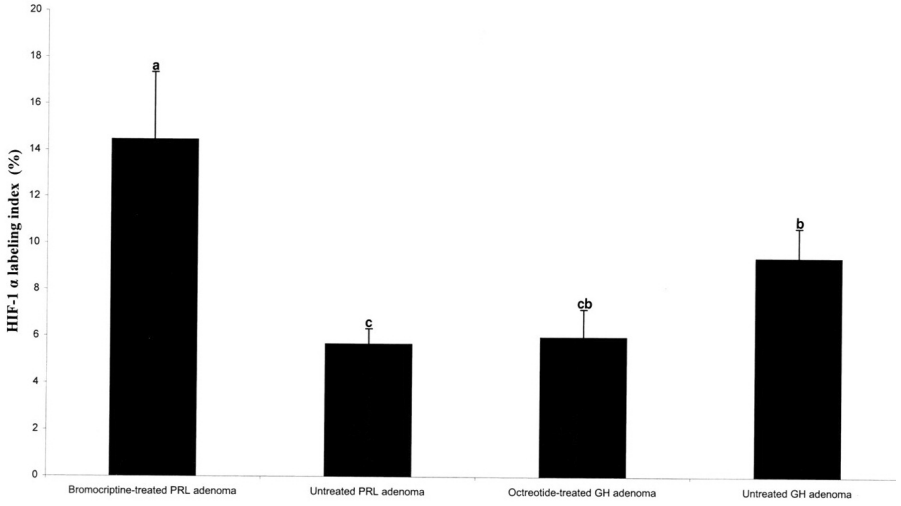

Fig. 6. HIF-1α labeling index in treated and untreated pituitary adenomas. The data are expressed as percentage of HIF-1α immunopositive cells and represent the mean ± SEM. Values with no letters in common are significantly different $p<0.05$ (statistical analysis with the Kruskall-Wallis analysis of variance and the Mann-Whitney U test).

microvessel density, orderly microvascular geometry contributes to increased cell proliferation activity in PRL-producing tumours (Vidal et al., 2003). Thus, microvascular density may not fully explain the interactions between vascular supply and pituitary tumour cell behaviour.

The lack of a relationship between HIF-1α expression and microvessel density in pituitary tumours may have an alternative explanation, one not dependent upon vascularity. For example, previous experiments have found that loss of HIF-1α expression in tumour cells renders them less vascular-dependent. Since activated oncogenes and mutated inactive tumour suppressor genes, such as PTEN (phosphatase and tension homolog detected on chromosome 10), VHL (von Hippel-Lindau tumour suppressor gene) and p53, can modulate HIF-1α levels in certain tumour types (Semenza, 1999), it may well be that any of these genes could modify HIF-1α expression in pituitary tumours. Mutation of p53 clearly confers a survival advantage to tumour cells when oxygen tension is very low (Lowe et al., 1993a,b; Wallace-Brodeur and Lowe, 1999; Ravi et al., 2000; Pluquet and Hainaut, 2001; Cadwell and Zambetti, 2001), and could contribute to the aggressive behaviour of some pituitary tumours. Indeed, it has been noted that mutation of p53 in pituitary tumours is accompanied by poor radioresponsiveness, increased invasiveness, and metastatic potential (Thapar et al., 1996; Suhardja et al., 1999). Although the expression of various oncogenes and tumour suppressor genes has been investigated in pituitary tumours (Suhardja et al., 1999; Heaney and Melmed, 2000; Yu and Melmed, 2001), more information is needed regarding the role of hypoxia in the regulation of HIF-1α expression.

Although in the present study HIF-1α expression showed no correlation with age, sex, tumour size, invasiveness, MIB-1 labeling, and microvessel density, determination of the HIF-1α index may provide useful information regarding possible role in up-regulating target genes that affect behaviour of pituitary tumours. Our study demonstrated an increase HIF-1α expression in bromocriptine-treated PRL-producing pituitary adenomas compared to untreated tumours. Dopamine agonists such as bromocriptine are principal therapeutic agents in the majority of patients with PRL-producing adenomas (Bevan et al., 1992; Molitch, 1999). They are effective in most cases, even in early phases of pituitary carcinoma therapy, but their action on pituitary cells is not completely understood. It is well known that dopamine agonists induce changes in PRL turnover and cell proliferation in PRL-producing tumours. However, the mechanism underlying the effect of these drugs in shrinking of pituitary tumours is unclear. Previous studies have not demonstrated a significant influence of dopamine agonists upon angiogenesis or apoptosis in PRL-producing adenomas. Since HIF-1α expression could affect both angiogenesis and p53 inducible apoptosis, its role in the modulation of bromocriptine response is an intriguing possibility worthy of further investigation.

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