IL-8 mRNA expression by *in situ* hybridisation in human pituitary adenomas

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Abstract

Several cytokines have been shown to be expressed in normal and adenomatous pituitary tissue. Recently, interleukin-8 (IL-8) mRNA was identified by reverse transcription (RT)-PCR in each of a series of 17 pituitary tumours examined. We have investigated further the presence of IL-8 mRNA, using *in situ* hybridisation in two normal human anterior pituitary specimens and 25 human pituitary adenomas. IL-8 mRNA was not identified in either of the two normal pituitary specimens. Only three of the 25 adenomas were positive for IL-8 mRNA. In these three tumours, which included two null cell adenomas and one gonadotrophinoma, the majority of tumour cells (>90%) were positive for IL-8 mRNA. The remaining 22 adenomas were completely negative. There was no difference in tumour size or type between the IL-8 positive and the IL-8 negative tumours, and immunocytochemistry for von Willebrandt factor showed that the two groups were also similar in their degree of vascularisation.

In conclusion, IL-8 mRNA was found in 12% of pituitary adenomas studied and was histologically identified within the tumour cells. *In situ* hybridisation is a more appropriate technique for assessing cytokine mRNA production by human pituitary tumours because RT-PCR may be too sensitive, identifying very small, possibly pathologically insignificant, quantities of mRNA that could be produced by supporting cells such as fibroblasts, endothelial cells or macrophages.

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Introduction

Cytokines are multi-functional peptides that are now known to be produced by a number of normal and neoplastic cells, apart from those of the immune system. Cytokines may act either systemically or locally within tissues as paracrine agents. Several cytokines have been shown to be produced by human pituitary adenomas and may have a part to play in the pathogenesis of these tumours (1, 2). Recently, expression of mRNA transcripts for several cytokines has been demonstrated in human pituitary tumours by reverse transcription (RT)-PCR (3, 4). These include the following cytokines: interleukins (IL)-1 α , -1 β , -2, -4, -6, -8, -10, -12, -14, -15, -16 and -17, tumour necrosis factors α and β , and transforming growth factor β . In addition, IL-1 receptor antagonist mRNA has been demonstrated in human corticotrophinomas, somatotrophinomas and non-functioning adenomas (5). The technique of RT-PCR, however, does not define the cells of origin, particularly whether or not the source is the neoplastic cell or other nonadenomatous cells such as fibroblasts, macrophages or endothelial cells. Immunoreactive and bioreactive

IL-6 is known to be secreted by between 50 and 60% of pituitary adenomas, independent of the tumour type (6). Moreover, the IL-6 mRNA has been demonstrated, by *in situ* hybridisation, to be present in the adenomatous cells of the pituitary tumours (6).

IL-8 is an inflammatory cytokine that was originally identified on the basis of its chemotactic activity (7). It was found initially in inflammatory cells, but has since been identified in some normal cells – for example thyrocytes – and in several neoplastic cells, including those of thyroid carcinoma, transitional cell carcinoma, squamous cell carcinoma, B-cell chronic lymphocytic leukaemia and melanoma (7). The functional role, if any, of IL-8 in these tumours has not been established, but there is some evidence that its production in the microenvironment of tumour cells *in vivo* could influence the immune response to the tumour by modulating the recruitment of neutrophils at the site of the tumour (7). Moreover, it has been suggested that IL-8 may act as an autocrine growth factor in melanoma cell lines (8), or may have a potent angiogenic effect similar to that of basic fibroblast growth factor (9).

IL-8 mRNA has been found consistently, by the very sensitive method of RT-PCR, in all pituitary adenomas in one study (3). A high number (35) of PCR cycles was required to demonstrate cytokine expression in pituitary adenomas, suggesting that a low number of mRNA transcripts were actually present. Contaminating non-tumour cells could potentially have contributed to the cytokine profiles found in that particular study. We have therefore used *in situ* hybridisation to look for IL-8 mRNA in pituitary adenomas. The technique of *in situ* hybridisation does allow the identification of the source of the mRNA using histological criteria (10).

Because IL-8 could potentially be involved in tumour angiogenesis, we performed immunocytochemistry for von Willebrandt factor (vWF), which assesses the degree of vascularisation of the tumours by identifying endothelial cells.

Materials and methods

Archival material from 25 pituitary adenomas removed transsphenoidally was used for this study. The tumours included ten null cell adenomas, five somatotrophinomas, five prolactinomas, four mixed growth hormone/prolactin adenomas and one gonadotrophinoma. Table 1 gives details of the patients and tumours. Two sections of normal anterior pituitary tissues were also studied.

In situ hybridisation for IL-8 mRNA

After deparaffinization and rehydration, sections were digested at 4 °C with proteinase K at a concentration of 10 µg/ml in Tris-HCl buffer for 10 min, followed by post-fixation in 0.4% paraformaldehyde in PBS, at 4 °C. Sections were then incubated in prehybridisation buffer for 60 min at 37 °C. The IL-8 antisense probe used was a cocktail of oligonucleotides labelled with fluorescein (R & D Systems Europe, Abingdon, Oxon, UK). Slides were hybridised to the probe diluted 1 : 100 in the buffer overnight at 37 °C, followed by washing in 4×SSC at 37 °C. After preincubation with 20% normal sheep serum, specifically bound probe was detected using anti-fluorescein-AP Fab fragments (Boehringer Mannheim, Lewes, UK) and, after a further wash with buffer, Nitra blue tetrazolium/5 bromo-4-chloro-3-indolyl-phosphate was used as chromogen. An inflamed appendix was used as a positive control and omission of the probe was used as negative control.

Immunocytochemistry for von Willebrandt factor

For the immunocytochemical detection of vWF, the avidin-biotin-peroxidase system (ABC) was used on formalin-fixed paraffin-embedded tissue sections. Briefly, the slides were dewaxed, rehydrated and treated

Table 1 Details of 25 patients with pituitary adenomas tested for IL-8 mRNA by *in situ* hybridisation (ISH).

Patient no.	Age (years)	Sex	Tumour size	Clinical presentation	Immunocytochemistry	IL-8 ISH
1	62	M	macro with sse	Prolactinoma	PRL	Negative
2	50	M	macro with sph se	Acromegaly	GH	Negative
3	55	F	large cystic macro	Non-functioning adenoma	Null	Negative
4	66	M	macro with sse	Non-functioning adenoma	Null	Negative
5	81	F	macro	Non-functioning adenoma	Null	Negative
6	44	F	macro with sse	Acromegaly	GH	Negative
7	32	M	large macro	Prolactinoma	PRL	Negative
8	63	F	macro with no sse	Acromegaly	PRL	Negative
9	35	M	macro with pse	Acromegaly	GH/PRL	Negative
10	50	M	macro with sse and pse	Acromegaly	GH	Negative
11	42	M	macro with sse	Acromegaly	GH	Negative
12	43	F	macro with sse	Acromegaly	GH/PRL	Negative
13	55	M	large cystic macro	Non-functioning adenoma	Null	Negative
14	74	M	macro with sse	Non-functioning adenoma	Null	Negative
15	46	M	macro with sse and cse	Prolactinoma	PRL	Negative
16	47	F	large cystic macro	Prolactinoma	Null	Negative
17	52	F	macro with sse	Non-functioning adenoma	Null	Positive
18	60	M	macro with sse	Non-functioning adenoma	GN	Positive
19	38	M	7-mm micro	Acromegaly	GH/PRL	Negative
20	46	F	macro with sse	Non-functioning adenoma	Null	Negative
21	74	M	macro with sse	Non-functioning adenoma	Null	Positive
22	61	F	macro	Acromegaly	GH/PRL	Negative
23	61	M	macro with sse	Prolactinoma	PRL	Negative
24	31	F	macro	Prolactinoma	PRL	Negative
25	54	M	huge macro with sse	Non-functioning adenoma	Null	Negative

macro, macroadenoma (i.e. >10 mm diameter); micro, microadenoma (i.e. <10 mm diameter); sse, suprasellar extension; sph. se, sphenoid sinus extension; pse, parasellar extension; cse, cavernous sinus extension; PRL, prolactinoma; GH, growth hormone-producing adenoma; GN, gonadotrophinoma.

with 3% hydrogen peroxide in methanol to inhibit endogenous peroxidase activity. Antigen retrieval was performed by incubation of the slides in 0.1% trypsin solution for 10 min. After the slides had been washed in Tris-buffered saline (TBS), normal goat serum was added, and after 15 min this was washed off by TBS and the slides were incubated for 30 min with anti-vWF rabbit polyclonal antibody (Dako, High Wycombe, UK) at a dilution of 1:400. After secondary biotinylated anti-goat antibody and ABC complex had been added, the antigen-antibody binding sites were visualised using the chromogen, diaminobenzidine. A colonic carcinoma section was used as a positive control, and omission of the antibody as a negative control.

Results

IL-8 mRNA was not identified in either of the two normal pituitary tissue specimens. It was identified in three pituitary adenomas, of which two were null-cell adenomas and the third the gonadotrophinoma. In each of these tumours, more than 90% of cells were clearly positive for IL-8 mRNA (Fig. 1). In the remaining 22 adenomas, no IL-8 mRNA was identified (Table 1). The three positive adenoma sections were examined by a pituitary neuropathologist and the IL-8 mRNA was localised to the adenoma cells. There was no evidence of increased numbers of inflammatory cells in the IL-8 positive sections examined, compared with the IL-8 negative ones.

Immunocytochemistry for vWF was performed on the three positive tumours and on five of the negative ones. Endothelial cells were identified by this technique in all eight tumours and, although there was variability in the degree of vascularisation between the different tumours and in different areas of the same tumour, there was no obvious difference in the degree of vascularisation between the IL-8 positive and the IL-8 negative tumours.

Discussion

This study has demonstrated, by *in situ* hybridisation, that IL-8 mRNA is present in the neoplastic cells in only a small proportion (12% in this study) of human pituitary adenomas. This finding is in contrast with the identification of IL-8 mRNA by RT-PCR being found in 100% of pituitary adenomas previously examined (3). *In situ* hybridisation appears to be a more appropriate investigation for the presence of cytokine mRNA transcripts in pituitary adenomas, as it is more likely to identify biologically significant expression (11). The high sensitivity of RT-PCR is likely to identify the negligible quantities of mRNA that may be present in non-neoplastic cells such as fibroblasts or occasional inflammatory cells.

In the three positive tumours, the IL-8 mRNA was identified predominantly within adenoma cells (Fig. 1).

Figure 1 (a) *In situ* hybridisation for IL-8 mRNA in a pituitary adenoma (patient No. 21 in Table 1), showing positive cytoplasmic staining (blue colour) for IL-8 mRNA in the majority of adenoma cells. (b) Negative control in the same adenoma with omission of the probe, showing only the green counterstain of the nucleus. Original magnifications, $\times 1200$.

On histological examination, none of the three IL-8 mRNA positive adenomas demonstrated aggressive or any other features pathologically or clinically different from those tumours that were negative (Table 1).

For a number of cytokines, mRNAs or synthesis and secretion of the protein, or both, have been demonstrated in human pituitary adenomas (1, 2). The expression of different cytokines, as for many other agents such as growth factors, varies between different types of pituitary adenomas. The pathophysiological role of the cytokines in pituitary adenomas is not apparent; however, cytokines in general have many actions, which include stimulation or inhibition of growth and proliferation, cellular differentiation, apoptosis and hormone secretion. In a minority of primary cultures of human pituitary adenomas, IL-6 in high concentration can have diverse effects on [^{3} H]thymidine uptake, with either stimulation or inhibition having been observed (12). A role for IL-8 in pituitary adenomas is not yet clear, as its main known function is as a neutrophil chemoattractant. Neutrophils are not found in any significant numbers within pituitary

adenomas and, in the tumours studied here, there was no increased number of polymorphonuclear cells in the positive adenomas, compared with those that were negative. IL-8 is known to be produced by monocytes, macrophages, T-lymphocytes, neutrophils, fibroblasts, endothelial cells and mesothelial cells (7). The production of IL-8 in the pituitary tumour microenvironment could potentially influence the immune response to the neoplastic cells by modulating the recruitment of neutrophils at the site of the tumour (7). Alternatively, IL-8 could have been produced by the adenoma cells as a response to hypoxic/anoxic insults on these cells after they had outgrown their rich blood supply, as has previously been shown for glioblastoma cells (13), suggesting a role for this cytokine in the promotion of angiogenesis. In support of this possibility is the fact that the three positive tumours were all large macroadenomas with suprasellar extension; however, they were not significantly different from the other 22 negative adenomas in size or type (Table 1). To assess further whether the IL-8 produced by the pituitary tumours was involved in the angiogenic process we performed immunocytochemistry for vWF on the three positive and on five negative tumours. Endothelial cells were identified by this technique in all eight tumours and, although different areas in the tumours showed variability in the degree of vascularisation, there was no difference between the IL-8 positive and the IL-8 negative tumours. This suggests that IL-8 in pituitary tumours is probably not involved in the stimulation of angiogenesis although the numbers examined were small.

No direct effects of IL-8 on pituitary hormone secretion have been reported, however, the cytokine-induced neutrophil chemoattractant, CINC/gro, a member of the IL-8 family, has been identified immunocytochemically and by Western blot analysis in the normal rat anterior pituitary (14). This cytokine has been shown to stimulate the production of prolactin and to inhibit secretion of luteinizing hormone and follicle-stimulating hormone by rat anterior pituitary cells (15).

The role of cytokines produced by human pituitary adenomas is not yet apparent. One hypothesis is that the production of different cytokine profiles by pituitary adenomas is an inadequate alarm signal by the abnormal cell to stimulate self destruction by apoptosis or to induce an immune response against itself. Other potential roles could be an involvement in modulation of hormone secretion or adenoma growth, through their effects on angiogenesis or other mechanisms.

In conclusion, IL-8 mRNA is expressed profusely by a minority of human pituitary adenomas. This study does show that *in situ* hybridisation may be a more appropriate investigation for the significant presence of cytokine mRNA transcripts in human pituitary tissue. The reason for IL-8 mRNA production in these tumours is as yet not clear, and requires further investigation.

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