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Constitutive (HO-2) and inducible (HO-1) haem oxygenase in pleomorphic adenomas of the human parotid: an immunocytochemical study

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Abstract

This study examines the expression HO-1 and HO-2 isozymes in human parotid pleomorphic adenomas. They are members of the heat shock protein family, and are thought to play a role in the regulation of tumoral blood flow. Immunocytochemistry using antibodies specific for HO-1 and HO-2 were undertaken in 12 pleomorphic adenoma specimens, all sections of which contained adjacent normal salivary tissue. Normal salivary gland acini and ducts displayed significantly stronger immunoreactivity for HO-2 compared to tumour cells ($p < 0.001$). Expression for HO-1 was minimal in both normal salivary gland acini and tumour cells with no difference ($p = 1.000$). However, positive staining for HO-1 was seen in normal salivary ducts and in pleomorphic adenomas showing ductal differentiation. In conclusion, this is the first study to examine the expression of HO-1 and HO-2 within normal salivary glands and pleomorphic adenomas. Our findings suggest that HO may be implicated in the pathogenesis of salivary pleomorphic adenomas.

Keywords: Adenoma; Pleomorphic; Parotid Neoplasms; Heat Shock Proteins; Haem Oxygenase

Introduction

The haem oxygenase (HO) protein enzymes are members of the stress/shock protein (HSP30) family which are responsible for the physiological oxidation and degradation of haem into carbon monoxide (CO), iron and biliverdin. Three isoforms have been identified to date: HO-1, HO-2, and HO-3.[1-3] HO-1 (HSP32), a heat shock protein, plays an essential role in cell response to oxidative and other cellular stresses. It is inducible in virtually all cell types by many cellular stress stimuli including hypoxia, cytokines and lipopolysaccharides. It protects cells from oxidative stress by its ability to reduce haem concentration, but also because the bile pigments produced have antioxidant properties.[4-6] Carbon monoxide and free iron are also biologically active. Carbon monoxide is involved in the generation of cyclic guanosine monophosphate (cGMP),[7] and it has been suggested that it acts as a signalling messenger molecule like nitric oxide (NO) in regulating vascular smooth muscle tone.[8] Free iron, on the other hand, regulates expression of HO-1, NO synthase, transferrin receptors and ferritin, as well as iron metabolism.[9-12] HO-2 is the constitutively expressed form of this enzyme and is unresponsive to any inducers of HO-1 with the exception of glucocorticoids.[13] Very little is known about HO-3. It lacks catalytic activity shared by the other two HO isoforms and it has been suggested that it acts as a binding protein of the haem molecule.[3]

Nitric oxide is an important gaseous mediator in the pathophysiology of tumorigenesis, in particular its involvement in the regulation of blood flow and the formation of new blood vessels.[14,15] In epithelial cells of the pleomorphic adenoma, expression of induced nitric oxide synthase (iNOS) was found to be significantly increased compared to normal salivary gland tissue.[16] In contrast to iNOS, the endothelial form (eNOS) is minimally expressed in both tumour and normal salivary tissue.

Nitric oxide is also known to induce HO-1 expression, and can inhibit HO-2.[13,17,18] Furthermore, HO-2 activity can inhibit functions of NO. Given this trans-inhibition relationship, and the role of both NO and CO in vascular dilatation and angiogenesis, the role of HO in tumorigenesis has been investigated in parallel to NOS in human and rat tumours.[19,20] Because of the potential role of HO in tumorigenesis, and the interactions between HO and NOS, the aims of the current study are therefore to

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examine the expression and distribution of HO-1 and HO-2 in normal salivary glands and in pleomorphic adenomas, and to determine whether there are any significant differences in the expression and distribution between normal and neoplastic salivary tissue.

Materials and methods

Twelve cases of pleomorphic adenomas of the parotid gland were selected from archival material obtained from the series of patients of one consultant head and neck surgeon (AMc). The tissues were fixed in 10 per cent formalin, sectioned serially through the tumour and embedded in paraffin, and were subsequently stained with haematoxylin and eosin. These sections were then reviewed by a consultant histopathologist (SDP) prior to immunostaining, both to confirm the diagnosis as well as to ensure that all sections contained adjacent normal salivary tissues which served as controls. All twelve cases fitted both criteria and were used for immunostaining.

Immunohistochemistry was performed on 6 μm sections using the streptavidin-biotin ABC technique with antibodies specific to HO-1 and HO-2. HO-1 expression was detected using a mouse monoclonal antibody (OSA110, Stressgen Biotechnologies Group, Victoria, British Columbia, Canada) at a dilution of 1:1000 for 25 min. HO-2 expression was detected using a rabbit polyclonal antibody (OSA-200, Stressgen Biotechnologies Group, Victoria, British Columbia, Canada) at 1:500 for 25 min. Signal was visualized using diaminobenzidine and sections counterstained with haematoxylin. Normal spleen which constitutively expresses HO-1, and normal testis which expresses HO-2, were used as positive controls for the respective isoforms.[13,21] Negative controls were stained in the absence of primary antibodies. The sections were analysed for HO staining positivity in a blinded fashion (both for the tumour and the antibody) by two independent observers (SDP and SL). Each case was given a score that is the sum of the scale of area of staining observed (at a magnification of x40) and the intensity of staining (viewed at a magnification of x200).[16] The area of staining was scored with a scale of 0 to 4: 0 = no staining of cells in any microscopic field; 1 = less than 25 per cent of tissue showing immunopositivity; 2 = between 25 per cent and 50 per cent immunopositivity; 3 = between 50 per cent to 75 per cent immunopositivity; 4 = greater than 75 per cent immunopositivity. The intensity of HO expression was on a scale of 0 to 3: 0 = no staining; 1 = mild staining; 2 = moderate staining; 3 = intense staining. The total score therefore ranged from 0 to 7. The results of the staining were analysed statistically with SPSS software. The Kolmogorov-Smirnov test was used to compare the staining in tumour cells with those in the normal gland itself.

Results and analysis

The mean age of the patients was 43 years (range 17-73 years), of whom seven were male and five were female. In all 12 specimens, the expression of HO-1 was noted in luminal cells of salivary ducts within normal salivary tissue. The intensity of staining for HO-1 was minimal (value 0) in normal gland parenchyma in all specimens except for three of the cases (values 2, 3, 3), but also minimal (value 0) in all the tumour epithelium except for four of the cases (values 2, 2, 3, 3). There was no significant difference in the staining of HO-1 in normal gland parenchyma compared to tumour (Figure 1; $p = 1.000$). In those pleomorphic adenomas in which the tumour showed significant ductal differentiation (eight of 12 cases), strong cytoplasmic staining for HO-1 within tumour ducts was seen. In contrast, the remaining area of the tumour showed only weak cytoplasmic staining. The latter was also observed in the myxoid (hypocellular) tumour for both HO-1 and HO-2; these represented five of the 12 cases. In three cases there was an

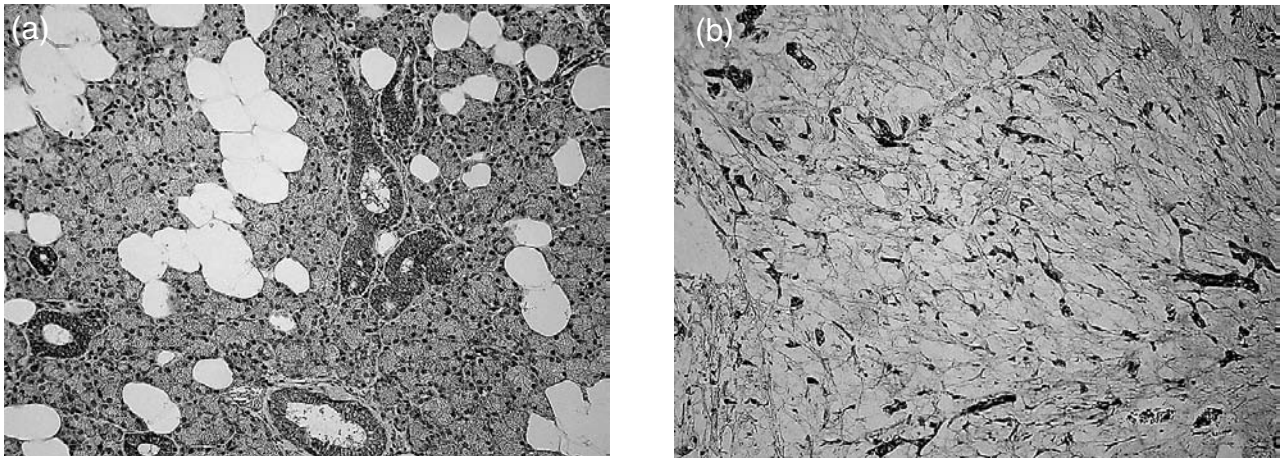

FIG. 1

(a) Normal salivary gland with intense staining of HO-1 in luminal cells of salivary ducts. (b) Pleomorphic adenoma myxoid tissue containing small ducts, showing strong staining for HO-1 within tumour ducts but not in the modified myoepithelial tumour cells. There was no significant difference in the staining of HO-1 in normal gland parenchyma compared to tumour ($p = 1.000$).

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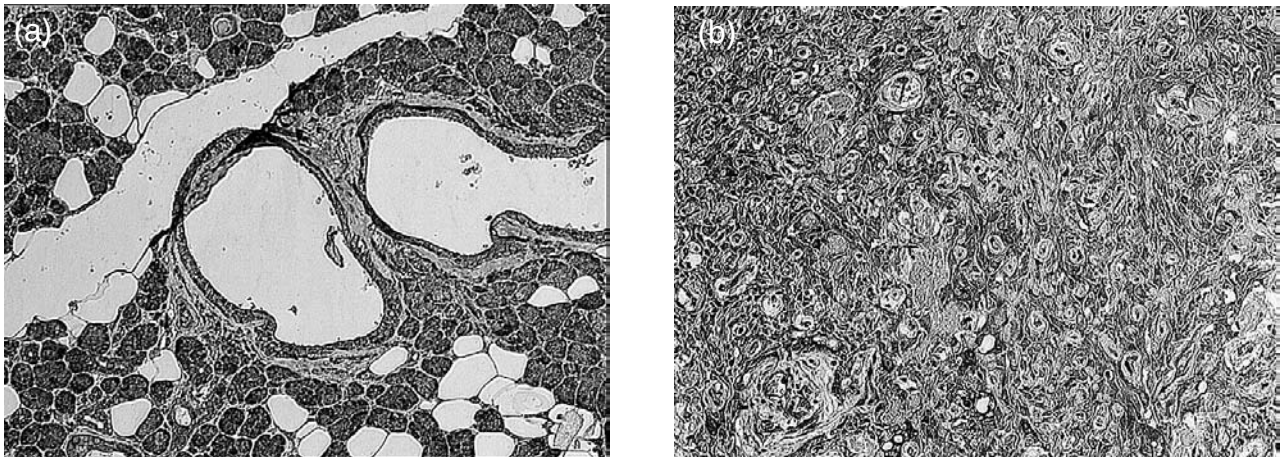

FIG. 2
(a) Intense staining of HO-2 in normal salivary gland acini and ducts but reduced in (b) pleomorphic adenoma showing a mixture of epithelial and myoepithelial cells, $p < 0.0001$.

intraparotid lymph node in the parotid gland, within which intense positive staining of HO-1 was observed in macrophages, but not in the adjacent mixed lymphoid population.

One of the slides stained for HO-2 was damaged and therefore excluded. Strong positive staining for HO-2 was detected in normal salivary gland ducts and acini in all 11 cases. Immunoreactivity for HO-2 was also observed in tumour cells in 10 of the 11 cases studied (Table I). Strong staining for HO-2 tended to be concentrated mostly in normal salivary ducts (11/11 cases) and ductal components of pleomorphic adenomas (6/11 cases). The intensity and proportion of staining in the normal salivary gland were much higher than that seen in the pleomorphic adenoma cells, and the difference was highly significant (Figure 2; $p < 0.0001$).

Discussion

This is the first study to report on the expression of haem oxygenase isoforms in the normal salivary gland and salivary pleomorphic adenoma. We hypothesize that the expression of the induced form of HO (HO-1) may be increased in the pleomorphic adenoma since NO induces HO-1 expression.[17] However the current study has demonstrated that, unlike iNOS, expression of HO-1 is minimal in both normal salivary gland tissue and epithelial cells of the pleomorphic adenoma with no difference in intensity. In addition, HO-1 immunoreactivity is significantly more intense in the neoplastic ducts within the pleomorphic adenoma. This observation seems to be highlighted by the weak positive staining observed in the surrounding modified myoepithelial cells. Our findings suggest that neoplastic ducts retain the ability to produce HO-1 and may be upregulated compared to normal salivary ducts, although further studies at a molecular level are required to confirm this.

By comparison, the constitutively expressed HO-2 also displays different distribution to eNOS. Intense immunoreactivity for HO-2 is observed in normal salivary gland acini and ductal epithelium, and is significantly reduced in tumour cells. Since iNOS expression is increased in the pleomorphic adenoma, and that it continues to produce NO for many hours and even days once induced, the release of NO in the pleomorphic adenoma was postulated to be much higher than the surrounding normal salivary tissue.[16] Previous studies in human glioma and cell lines have shown that NOS and HO are generally not mutually expressed.[18] Given that NO inhibits HO-2 expression,[17,18] and that iNOS expression is increased in epithelial cells of the pleomorphic adenoma,[16] we postulate that the reduced expression of HO-2 in the pleomorphic adenoma may be a result of the ability of NO to suppress HO-2 expression. This finding is consistent with previous study in the experimental C6 striatal glioma model.[20] NO stimulates angiogenesis and tumour blood flow.[14,15] Like NO, expression of HO-1 mRNA correlates with macrophage infiltration and vascular density in human malignant glioma.[19] In the current study, however, the expression of HO isoforms in blood vessels in the pleomorphic adenoma has not been demonstrated, although HO-1 was detected in macrophages.

TABLE I
COMPARISON OF EXPRESSION OF HO-2 IN NORMAL PAROTID TISSUE AND PLEOMORPHIC ADENOMA

Case No.	Normal salivary gland (excluding ducts)	Pleomorphic adenoma
1	7	2
2	7	3
3	5	5
4	7	2
5	7	5
6	6	3
7	6	3
8	6	4
9	6	2
10	7	4
11	6	5
12 (excluded)		

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PLAG1 and PLAGL2 are members of the PLAG (pleomorphic adenoma gene) family.[22] Ectopic expression of PLAG1 on chromosome 8q12 is present in the largest cytogenic subgroup of the salivary pleomorphic adenoma,[23] and it has been suggested to lead to the expression of the target gene involved in tumorigenesis of this tumour.[24,25] In contrast, PLAGL2, which is markedly induced under hypoxic and iron depletion conditions, was postulated to regulate the expression of the iron depletion-inducible gene.[26] It has also been suggested that it serves as a tumour suppressor gene by its involvement in cell cycle arrest and by apoptosis of tumour cells through hypoxia-induced activation of genes encoding the pre-apoptic proteins of the Bcl-2 family.[27] Whether locally altered HO expression in the pleomorphic adenoma and the subsequent disruption of local oxygen tension and iron level bears any relationship to PLAGL2 expression remains speculative.

- **This is a preliminary study examining the expression of haem oxygenase proteins in parotid pleomorphic adenomas**
- **Pleomorphic adenomas showed down-regulation of HO-2 compared to normal tissue**
- **HO-1 levels were increased in both normal ducts and in tumours where ductal differentiation was a prominent feature**
- **The authors conclude that further work is needed to gain a better understanding of the role of these haem oxygenase proteins in the genesis of these tumours**

Conclusion

The current study constitutes the first report to detect HO-1 and HO-2 in the normal human parotid gland as well as in the pleomorphic adenoma. Our findings showed downregulation of HO-2 expression in the pleomorphic adenoma, and increased expression of HO-1 in normal salivary gland ducts and also in those pleomorphic adenomas in which ductal differentiation was a prominent feature of the tumour. The role of HO in the pathogenesis of pleomorphic adenomas remains to be established. Future work in this area may lead to better knowledge of the role of HO in the tumorigenesis of this tumour.

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