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Main Article

Expression of cluster of differentiation 9 glycoprotein in benign and malignant parotid gland tumours

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Abstract

Objectives: This study aimed to clarify the significance of cluster of differentiation 9 glycoprotein gene expression in human parotid gland tumours.

Methods: We retrospectively analysed immunohistochemical staining for cluster of differentiation 9 glycoprotein in parotid gland tumours.

Results: Cluster of differentiation 9 glycoprotein was consistently detected in the normal parotid gland. Regarding benign parotid gland tumours, cluster of differentiation 9 glycoprotein was present in 13 of 18 pleomorphic adenomas, in all Warthin tumours tested (21/21) and in all cases of basal cell adenoma tested (four of four). In contrast, positive staining for cluster of differentiation 9 glycoprotein was less often observed in malignant parotid tumours. Cluster of differentiation 9 glycoprotein was present in 11 of 14 mucoepidermoid carcinomas, in two of five acinic cell carcinomas and in two of five adenoid cystic carcinomas.

Conclusions: There was a statistically significantly reduced expression of cluster of differentiation 9 glycoprotein in malignant parotid gland tumours, compared with benign parotid gland tumours ($p < 0.05$). These results suggest that a low level of cluster of differentiation 9 glycoprotein expression in parotid gland tumours may be associated with malignancy.

Key words: Parotid Gland Tumors; CD9; Immunohistochemistry

Introduction

Cluster of differentiation 9 glycoprotein antigen has a molecular weight of 24–27 kDa and was first identified as a human B lymphocyte differentiation antigen.[1] This glycoprotein was initially reported to be expressed in pre-B cells and platelets, but is now known to also be expressed in a wide variety of haematopoietic and nonhaematopoietic cells. Cluster of differentiation 9 glycoprotein is identical to diphtheria toxin receptor associated protein[2] and motility-related protein 1.[3] Our previous studies on diphtheria toxin receptor associated protein showed that cluster of differentiation 9 glycoprotein forms a complex with the diphtheria toxin receptor, and that this complex is identical to membrane-anchored heparin-binding epidermal growth factor like growth factor.[4] We have also shown that cluster of differentiation 9 glycoprotein up-regulates sensitivity to diphtheria toxin and also increases the mitogenic effect of membrane-anchored heparin-binding epidermal growth factor like growth factor on neighbouring cells.[5] Cluster of differentiation 9 glycoprotein belongs to the transmembrane-4 superfamily, members of which are characterised by four trans-membrane domains, two extracellular loops, and intracellular N- and C-termini.

One characteristic feature of cluster of differentiation 9 glycoprotein, and of transmembrane-4 superfamily members in general, is the ability to form complexes with other membrane proteins, for example with diphtheria toxin receptor (to form membrane-anchored heparin-binding epidermal growth factor like growth factor) and with integrin $\alpha 3\beta 1$ and $\alpha 6\beta 1$.[6] Therefore, the biological function of cluster of differentiation 9 glycoprotein in normal and cancer cells could be expected to be complex. Cell adhesion to extracellular matrix proteins may be stimulated by cluster of differentiation 9 glycoprotein.[7] Transfection of cluster of differentiation 9 glycoprotein copy deoxyribonucleic acid (DNA) into cultured tumour cells suppresses cell motility and growth *in vitro*.[3] Several studies have shown that cluster of differentiation 9 glycoprotein regulates cell motility by either enhancing[8] or suppressing[3] cellular migration. Studies of lung and breast cancer have found decreased expression of cluster of differentiation 9 glycoprotein to be associated with the tumour's metastatic potential.[9,10]

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In the present study, we analysed cluster of differentiation 9 glycoprotein expression in human parotid gland tumours, using immunohistochemical staining. We also compared the expression and distribution of cluster of differentiation 9 glycoprotein within human parotid gland tumours.

Methods

Sample collection

Surgical specimens of parotid gland tumours and normal parotid glands (from a total of 67 patients) were obtained from the department of otolaryngology and head and neck surgery, Kurume University Hospital. The patients comprised 31 men and 36 women, and had a mean age of 65.3 years. Patients' histopathological diagnoses comprised 43 benign tumours and 24 malignant tumours, as follows: 18 pleomorphic adenomas, 21 Warthin's tumours, four basal cell adenomas, 14 mucoepidermoid carcinomas, five acinic cell carcinomas and five adenoid cystic carcinomas. None of the patients had undergone pre-operative radiotherapy or chemotherapy.

From the paraffin-embedded blocks of the resected tumours, 5-mm thick sections were cut, conventionally deparaffinised by xylene, dehydrated by ethanol and immunohistochemical staining for cluster of differentiation 9 glycoprotein was done. Several normal parotid glands were used for control staining.

Antibody preparation

Anti cluster of differentiation 9 glycoprotein monoclonal antibody 007 was prepared and purified as previously described.[2]

Immunohistochemical staining

The paraffin-embedded sections were sequentially incubated at room temperature with diluted primary antisera for 20 minutes, 3 per cent hydrogen peroxide for 5 minutes, biotinylated secondary antisera (rabbit, mouse, goat, sheep or rat; BioGenex, San Ramon, California, USA) for 20 minutes and strept-avidin-enzyme conjugate (BioGenex) for 20 minutes. Each incubation was followed by three 5-minute washes in phosphate-buffered saline. The sections were exposed to the chromogen reaction solution (3-amino-ethyl carbazole; Lipshaw, Detroit, Michigan, USA) for 10 minutes. After washing in tap water, the specimens were briefly counterstained.

The primary antibody (i.e. 007 against cluster of differentiation 9 glycoprotein) was diluted to 1:20. Primary antibodies absorbed with excess antigenic peptides or normal serum, or omitted altogether, were used as controls.

Determination of cluster of differentiation 9 glycoprotein expression

The degree of cluster of differentiation 9 glycoprotein expression in the parotid gland was determined by dividing cells into two groups: either negative, indicating no cluster of differentiation 9 glycoprotein staining throughout the specimen; or positive, indicating cluster of differentiation 9 glycoprotein staining throughout the specimen. Decisions on the type of staining pattern were reached by consensus between four different observers. Cluster of differentiation 9 glycoprotein staining was performed and compared for each tumour and for normal parotid gland tissue.

Differences in cluster of differentiation 9 glycoprotein expression between benign and malignant tumours were statistically analysed using Fisher's exact probability test. A value of $p < 0.05$ was considered significant.

Results

Expression in normal parotid glands

Immunohistochemical staining of normal parotid gland tissue with anti cluster of differentiation 9 glycoprotein monoclonal antibody (MAb) revealed that the glycoprotein was distributed mainly in the duct luminal cells (Figure 1a); peripheral nerve fibres were also positively stained. The most intensive staining was found in the basal cells of the striated duct and the excretory duct. Acinic cells, myoepithelial cells and fat cells did not stain for cluster of differentiation 9 glycoprotein.

Expression in parotid gland tumours

The results of immunohistochemical staining for each parotid gland tumour type are summarised in Table I.

In pleomorphic adenomas, expression of cluster of differentiation 9 glycoprotein was seen in some intercalated duct cells (to a slight degree) and also in regions with extensive squamous differentiation (Figure 1b). There was no expression of cluster of differentiation 9 glycoprotein in the myxoid stroma or luminal cells. Thirteen of 18 pleomorphic adenomas showed positive immunoreactivity. In Warthin's tumours, cluster of differentiation 9 glycoprotein expression was found in all cases, and intense staining was seen in 15 cases. Cluster of differentiation 9 glycoprotein expression was found in the intercellular portion of the intercalated duct (Figure 1c). Various degrees of staining were seen in the striated duct cells and luminal and basal cells. No staining was observed in the lymphocytes or myoepithelial cells of the interstitial tissue. In the four basal cell adenomas, cluster of differentiation 9 glycoprotein staining was observed in the basal cell and luminal cell components, but not in the myoepithelium (Figure 1d).

In the 14 mucoepidermoid carcinomas, positive cluster of differentiation 9 glycoprotein immunoreactivity was detected in 11 cases, and was intense in six of these. Staining was observed in the mucous acinar cells (Figure 2a). In the five acinic cell carcinomas, cluster of differentiation 9 glycoprotein positivity was found in the solid variant of acinic cells in two cases (Figure 2b). In the five adenoid cystic carcinomas, cluster of differentiation 9 glycoprotein positivity was found in the solid variant of neoplastic basal cells in two cases (Figure 2c).

The relationship between cluster of differentiation 9 glycoprotein expression and parotid gland tumour

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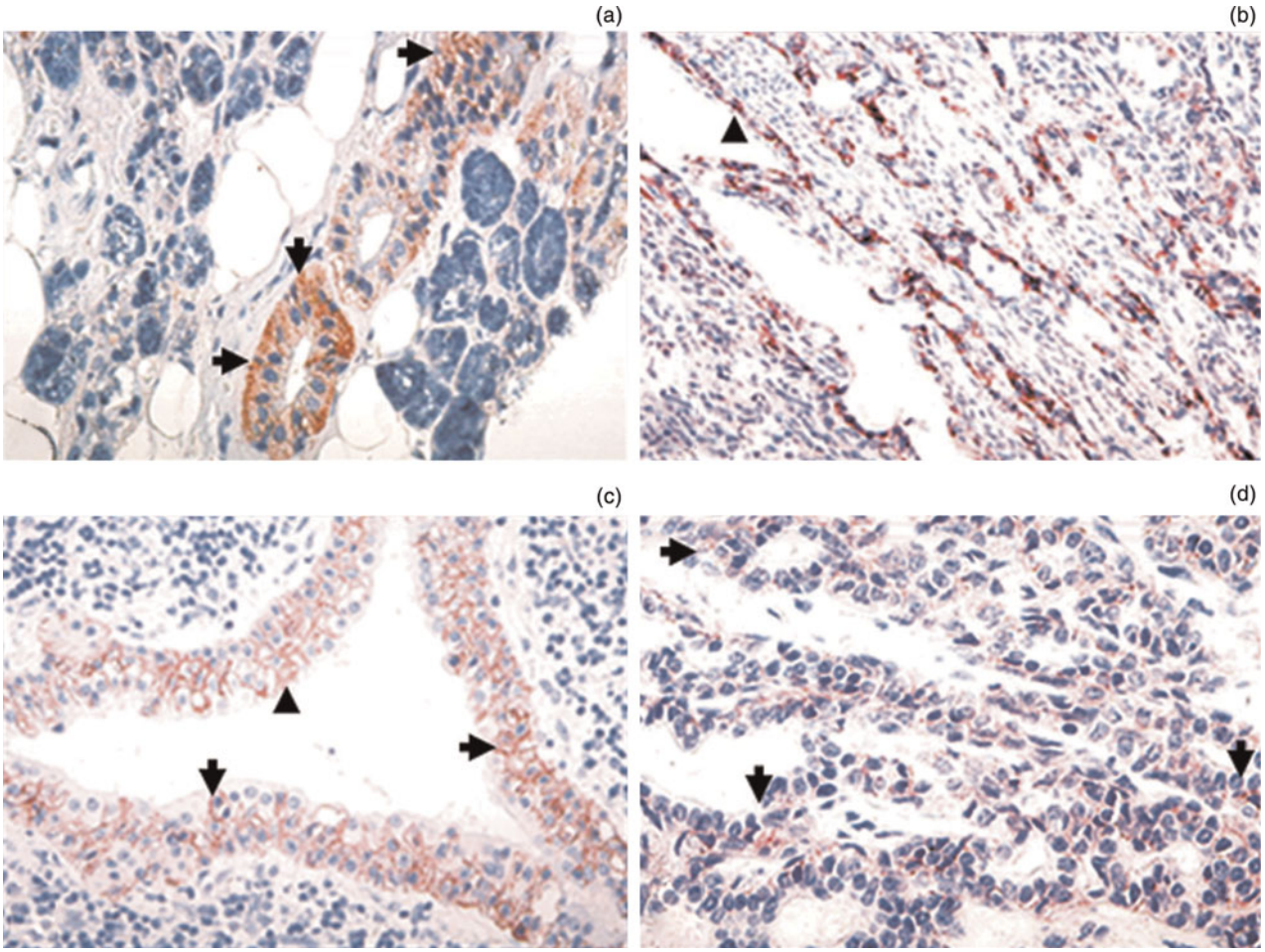

FIG. 1

Photomicrographs showing immunohistochemical staining for cluster of differentiation 9 glycoprotein. (a) Normal parotid gland, showing intensive staining in the basal cells of the striated duct and the excretory duct (arrows); no staining was seen in the acinic, myoepithelial or fat cells. (b) Pleomorphic adenoma, showing slight staining in the intercalated duct cells (arrowhead). The arrowhead is strong expression of CD9. (c) Warthin's tumour, showing staining in the intercellular portion of the intercalated duct (arrows); lymphocytes in the interstitial tissue showed no staining. (d) Basal cell adenoma, showing staining in basal cells and luminal cells (arrows) but not in myoepithelial cells. (Original magnification ×100)

malignancy is summarised in Table II. In the 43 benign tumours, cluster of differentiation 9 glycoprotein expression was observed in 38 (88 per cent) cases. In contrast, only 15 (63 per cent) of the 24 malignant tumours were positive for cluster of differentiation 9 glycoprotein. Therefore, cluster of differentiation 9 glycoprotein expression was more often seen in benign parotid gland tumours than in malignant tumours ($p = 0.029$). However, no correlation was observed between cluster of differentiation 9 glycoprotein expression and grade of tumour malignancy.

TABLE I

IMMUNOHISTOCHEMICAL STAINING FOR CD9 IN PAROTID GLAND TUMOURS

Tumour	Staining pattern	
	−ve	+ve
Benign		
Pleomorphic adenoma	5	13
Warthin's tumour	0	21
Basal cell adenoma	0	4
Malignant		
Mucoepidermoid Ca	3	11
Acinic cell Ca	3	2
Adenoid cystic Ca	3	2

Data represent number of cases. CD9 = cluster of differentiation 9 glycoprotein; −ve = negative; +ve = positive; Ca = carcinoma

Discussion

The gene for cluster of differentiation 9 glycoprotein (also know as motility related protein 1) encodes a 24–27 kDa glycoprotein that contains four hydrophobic domains and an extracellular N-glycosylated domain; the latter may function as a cell surface anchored receptor. Cluster of differentiation 9 glycoprotein was originally characterised as a cell surface antigen on lympho-haematopoietic cells. Pre-B cells and platelets are known to express relatively large amounts of cluster of differentiation 9 glycoprotein.[1] Anti cluster of differentiation 9 glycoprotein antibody induces pre-B cell adhesion to bone marrow fibroblasts, mediated by very late antigen (VLA)

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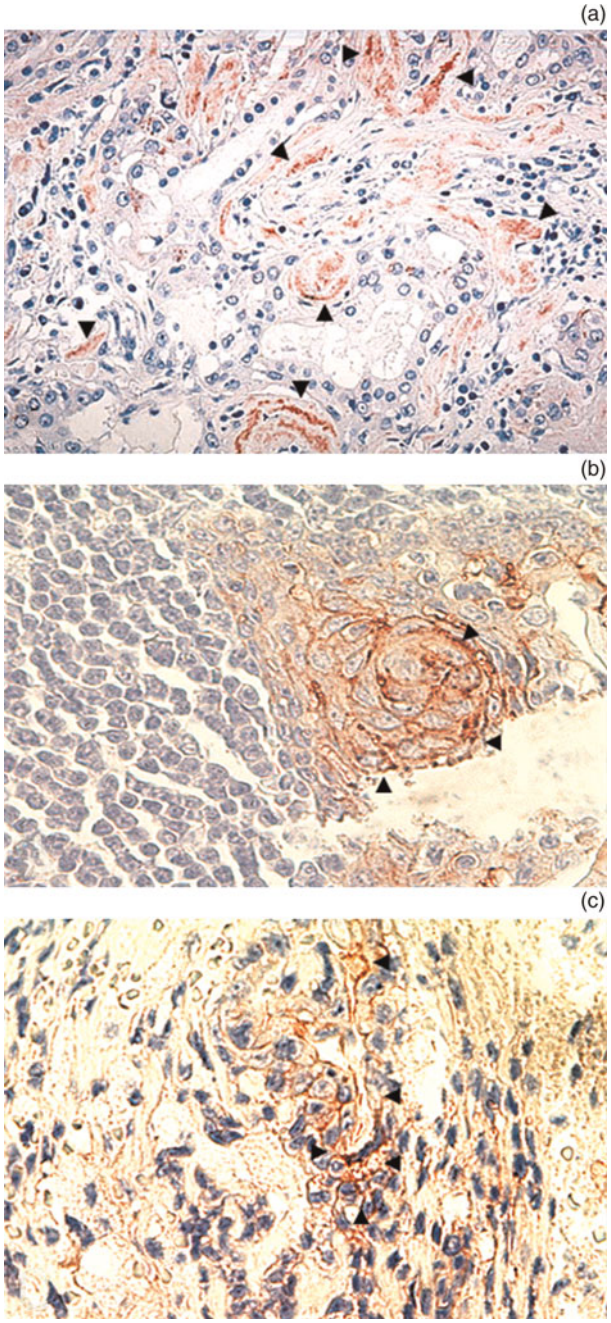

FIG. 2

Photomicrographs showing immunohistochemical staining for cluster of differentiation 9 glycoprotein. (a) Mucoepidermoid carcinoma, showing staining in mucous acinar cells (arrowheads), with granules of cluster of differentiation 9 glycoprotein in the cytoplasm. (b) Acinic cell carcinoma, showing staining in the solid variant of acinic cells (arrowheads). (c) Adenoid cystic carcinoma, showing staining in a solid variant of neoplastic basal cells (arrowhead). (Original magnification ×100)

4 and 5 integrins, thus suggesting that cluster of differentiation 9 glycoprotein is involved in the integrin-regulated cell adhesion process.[7] Recently, diphtheria toxin receptor associated protein and motility-related protein 1 (both alternative names for cluster of differentiation 9 glycoprotein) have been independently identified in non-haematopoietic cells. Diphtheria toxin receptor-associated protein, first

TABLE II

IMMUNOHISTOCHEMICAL STAINING FOR CD9 IN PAROTID GLAND TUMOURS: SUMMARY

Tumour	Staining pattern		Total (*n*)	*p*
	−ve	+ve		
Benign	5	38	43	0.029
Malignant	9	15	24	

Data represent number of cases. CD9 = cluster of differentiation 9 glycoprotein; −ve = negative; +ve = positive; Ca = carcinoma

identified in Vero cells,[2] is the monkey homologue of cluster of differentiation 9 glycoprotein.[11] Motility-related protein 1, originally identified as an antigen the antibody of which was known to inhibit the motility of several carcinoma cell types, is also identical to cluster of differentiation 9 glycoprotein.[3]

Cluster of differentiation 9 glycoprotein has been shown to form complexes with integrin α3β1 and with integrin α6β1;[6] thus cluster of differentiation 9 glycoprotein may affect cell motility through interaction with integrins. Integrin α3β1 was first identified as a tumour-specific antigen, and has shown enhanced expression in some kinds of tumour cells. Integrin α3β1 was localised at cell substratum attachment sites in some cells, but it has also often been observed at intercellular attachment sites in epithelial cells.[12] Although the biological function of integrin α3β1 is unclear, cooperation of integrin α3β1 with cluster of differentiation 9 glycoprotein may be necessary to maintain epithelial cells in their normal state. Loss of cluster of differentiation 9 glycoprotein may thus result in unregulated motility or invasion mediated by integrins.

Recent studies of clinical samples of human cancers have demonstrated a relationship between reduced expression of cluster of differentiation 9 glycoprotein and aggressive tumour behaviour. Some studies of cluster of differentiation 9 glycoprotein have detected it in various cell types, and authors have theorised that cluster of differentiation 9 glycoprotein expression may suppress cell motility and tumour metastasis.[13] Cluster of differentiation 9 glycoprotein belongs to the transmembrane-4 superfamily, members of which have four highly conserved hydrophobic domains, which are assumed to span the lipid bilayer of the cell membrane.[14] The transmembrane-4 superfamily consists of around 15 member proteins which are variously expressed by leukocytes and a variety of mammalian tissues, as well as on the surface of two types of parasites. The precise physiological functions of these proteins remain unknown; however, their respective genes' DNA has been highly preserved throughout evolution, suggesting that the genes play an important role. Several possible functions have been reported, including participation in signal transduction, antigen presentation, cell proliferation, cell adhesion and cell motility.[10,15]

In addition to cluster of differentiation 9 glycoprotein, the expression of two other members of the

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transmembrane-4 superfamily have been shown to correlate with metastasis. Cluster of differentiation 82 glycoprotein, also known as Kangai 1 (KAI1) (suppression of tumorigenicity 6, prostate: CD82 antigen (R2 leukocyte antigen, antigen detected by monoclonal and antibody IA4)), has been identified as a metastasis suppressor gene for prostate cancer, and its expression has been observed to down-regulate during progression of human prostate cancer.[16] Another transmembrane-4 superfamily member, cluster of differentiation 63 glycoprotein, also known as ME491 (melanoma-associated glycoprotein family. Antigenic identity of the ME491, NKI/C-3, NGA and CD61 proteins), has been identified in human melanoma cells and is preferentially expressed in the early stages of tumour progression; however, its expression declines significantly and sometimes disappears entirely in advanced, rapidly growing melanomas and metastatic melanoma cells.[17] A recent study has disclosed the existence of a transmembrane-4 superfamily network on the cell surface, further suggesting a close correlation among cluster of differentiation 9, 63 and 82 glycoproteins.[15]

In the literature, epidermal growth factor, heparin-binding epidermal growth factor, epidermal growth factor receptor, Erb B4, tumour growth factor β2, and tumour growth factors βR I and II have been reported to be present mainly in ductal cells, based on immunohistochemical analyses of the normal parotid gland.[18] In the same way, integrin α3β1 and β3 have been found to be positive mainly along the basal lamina of the ductal cells and in the stroma in the normal parotid gland. Warthin's tumour is a unique neoplasm arising almost exclusively in the parotid gland. It consists of bilayered luminal and basal epithelia and follicle-containing lymphoid tissue. The pathogenesis of this tumour has been variously discussed, and immunohistochemical studies using various antibodies have been reported.[19] Epithelial and/or lymphoid cells within Warthin's tumour have also been found to express integrins (such as α3β1 and β3), (Thy-1 CD90 (Cluster of Differentiation 90) is a 25–37 kDa heavily N-glycosylated, glycophosphatidylinositol (GPI) anchored conserved cell surface protein with a single V-like immunoglobulin domain, originally discovered as a thymocyte antigen), cluster of differentiation 44 glycoprotein and VCAM-1 (vascular cell adhesion molecule-1, also known as CD106, is a molecule with a considerable role in the human immune system).[18] Between themselves, these various proteins are thought to regulate the proliferation and cell attachment of both epithelial and lymphoid components of Warthin's tumour. Expression of cluster of differentiation 9 glycoprotein has been observed in the luminal and basal epithelia of this tumour. As a result, cluster of differentiation 9 glycoprotein has been assumed to form integrin α3β1 in Warthin's tumour.

A significant reduction in or complete loss of cluster of differentiation 9 glycoprotein expression was observed in oral squamous cell carcinoma, at the periphery of the cancer nests in the advancing front of the invading tumour.[20] Loss of cluster of differentiation 9 glycoprotein expression in oral squamous cell carcinoma strongly correlated with a high incidence of cervical lymph node metastasis and a poorer prognosis. In the present study, the expression of cluster of differentiation 9 glycoprotein was comparatively greater in benign tumours and less in malignant tumours. Such general tendencies were observed by investigating a large number of cases, of varying tumour types.

Further study is required to clarify the precise mechanism of cluster of differentiation 9 glycoprotein gene expression in cancer progression and metastasis.

Conclusion

This study found a relationship between deterioration of parotid gland tumours and reduced expression of cluster of differentiation 9 glycoprotein. Distribution of CD9 glycoprotein in parotid gland and parotid gland tumors was observed to be similar to that in the intercalated duct cell in parotid gland tumours, in paraffin-embedded specimens. Immunohistochemical studies using paraffin-embedded specimens showed preferential localisation of cluster of differentiation 9 glycoprotein in the normal parotid gland and in parotid gland tumours.

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