The ribosomal P0 protein induces a spontaneous immune response in patients with head and neck advanced stage carcinoma that is not dependent on its overexpression in carcinomas

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Abstract. A typical feature in systemic lupus erythemathosus patients is the presence of autoantibodies to the carboxyl-terminal homologous P proteins (P0, P1, P2) domain (C-22 P0 epitope). In this report we provide evidence for the *in vivo* immunogenicity of the P0 protein in head and neck cancer patients as well as overexpression by immunohistochemistry of the C-22 P0 epitope in invasive carcinomas (55/57). Overexpression of this epitope was also significantly associated with a number of pathological lesions arising in the head and neck stratified epithelium including acanthosis (8/8), benign tumors (11/11), dysplasia (23/25) and *in situ* carcinomas (9/9). Intermediate cell layer restricted epitope overexpression was observed in well differentiated carcinomas, while undifferentiated tumors showed overexpression throughout the cell layers. Employing recombinant P proteins, sera from 40 of the 57 carcinoma patients and 39 normal donors, were subjected to immunoblot analysis. Immunity to P0 protein (7/40) was associated with malignancy and with advanced disease stage, but it was not dependent on the C-22 P0 epitope overexpression, although it was the preferential autoantibody target in 4 patients. Increased expression of the C-22 P0 epitope on the surface of pharynx cancer cells following cellular stress *in vitro*, may imply P0 protein presentation as an *in vivo* autoantibody target in cancer patients. Evidence for immunity to the P0 protein, as well as overexpression in patients with head and neck carcinoma may be relevant for monitoring cancer progression, in planning immunotherapeutic strategies, and contribute to the understanding of immuno-biological behaviour of head and neck carcinomas.

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Introduction

Head and neck squamous cell cancer (HNSCC) incidence is increasing worldwide and despite advances in cancer treatment, the survival rate of patients with this type of cancer has not substantially changed over the last two decades (1). The development of HNSCC is a multistep process arising from precancerous lesions that progress into malignant tumors (2-4). The identification of molecular targets is essential for improving diagnostic and therapeutic strategies for HNSCC management. Among others, antigens overexpressed by malignant tumors represent suitable immunological targets and, when also overexpressed in premalignant lesions might help identify lesions with high risk of malignant transformation (5). To date, several methods have been employed to identify in HNSCC, targets for potential diagnostic and/or therapeutic use (6-9). In addition, antibodies derived from animals immunized with cancer cells are currently being used to identify tumor target antigens (10,11). Here, we provide evidence for the *in vivo* spontaneous immunogenicity of the ribosomal P0 protein in head and neck cancer patients as well as for the differential expression of its carboxyl-terminal epitope, detected by a novel monoclonal antibody (MAb 2B2), in a number of pathological lesions arising in the head and neck stratified epithelium compared to normal epithelium.

The ribosomal P proteins (P0, 38 kDa; P1, 19 kDa and P2, 17 kDa) constitute a pentameric complex forming the ribosomal stalk of the 60 S ribosomal subunit in the eukaryotic cells (12). P0 exists as a free protein in the cytoplasm and on the surface of cancer cells (13,14). Several studies have demonstrated the presence of autoantibodies to ribosomal P proteins, in sera of patients with systemic lupus erythematosus (LES) (15-21). A 22 amino acid P protein common carboxyl-terminal epitope (C-22 P0 epitope), turned out to be the preferential target of the autoantibodies (15-21). Moreover, the level of ribosomal P0 protein is regulated during development and apoptosis (22,23). Only few reports describe overexpression of ribosomal P proteins in human carcinomas (24,25). To our knowledge no report exists regarding the immunogenicity of the P proteins in cancer patients. Evidence for immunity to the P proteins, as well as overexpression in HNSCC, may be relevant for monitoring cancer progression, in planning immunotherapeutic strategies targeting these proteins and also contribute to the understanding of immunobiological behaviour of HNSCC.

Materials and methods

Cell lines. Head and neck cancer cell lines derived from tongue (SCC-15, CAL 27, CAL 33) or pharynx (FaDu) as well as mesothelioma (26), lung (A549), breast (MCF-7) and ovary (OVCAR) cancer cell lines were maintained in RPMI containing 10% fetal bovine serum.

Generation of monoclonal antibody (MAb) 2B2. Four-weeks old Balb/c mice were immunized twice by intra-peritoneal (i.p) injection with $2x10^6$ FaDu cells in 500 μl phosphate-buffered saline solution (PBS) and boosted intravenously with $1x10^4$ cells. Somatic cell hybrids were prepared using the mouse myeloma cells line NS-1 as previously described (27). MAb 2B2 was purified by protein G (Sigma-Aldrich, St. Louis, MO).

Enzyme linked immunosorbent assay (ELISA). MAb 2B2 immunoreactivity was determined using $5x10^4$ cells/well or peptides as previously described (28). The P proteins common carboxyl-terminal 22 amino acid peptide (C-22 P0 epitope) (NH_2-AKVEAKEESEESDEDMGFGLFD-COOH) was synthesized by Primm (Primm, Italy) (15-17,20). The ErbB4 283 peptide (Santa Cruz Biotech, CA) was used as control peptide. ELISA plates (Pro-bind™ , Falcon, NJ) were coated overnight with 1-5 μg/well of the peptides. Plates were incubated after blocking with MAb 2B2 or MOPC-21 (Cappel, Organon Teknicka, PA, USA) (1 μg/ml) or human sera (1:100). The 1:100 dilution was chosen since it was the highest serum concentration lacking background reactivity.

Identification of the antigen recognized by MAb 2B2. Biphasic mesothelioma cells ($2x10^7$) were used to identify the protein recognized by MAb 2B2 (29). Briefly, mesothelioma cell extract was submitted to two-dimensional (2DE) electrophoresis (sample A, 70% of the total) and two-dimensional electrophoresis following Western blotting using MAb 2B2 (sample B, 30% of the total) as probe. After 2DE separation, gel containing sample A was stained with Sypro Ruby gel stain (Molecular Probes) and a digital image was recorded. Proteins from sample B were blotted to Immobilon-NC membranes (Millipore, Bedford, MA, USA) and after staining with Sypro-Ruby Blot stain (Molecular Probes) the membrane image was recorded. After blocking, membrane was incubated overnight at 4˚C with MAb 2B2. Western blotting image was matched with the Ruby-stained image by using the Bio-Rad PDQuest software (version 7.1, Bio-Rad Laboratories, Milano, Italy). The immuno-reactive protein was identified by submitting to proteolysis and MS/MS analysis the matching spot excised from gel. Protein digestion and mass-spectrometric analyses were performed by Swiss 2DSERVICE (Geneva Proteomics Research Centre, Switzerland) (29). Peptide fragmentation sequencing data were analyzed for similarity search in Swiss-Prot, TrEMBL, and NCBInr protein databases, using Mascot software.

Molecular cloning and recombinant expression of P0, P1 and P2 ribosomal proteins. Total RNA extracted from FaDu cells was retrotranscripted using each downstream P0, P1 and P2 primers with the respective sequences 5'-TTAGTCAAA GAGACCAAATCCCATATC-3' (NM_001002), 5'-TTAGC CTCTGTCTCCGAGCTCG-3' (NM_001003) and 5'-TTAAT CAAAAAGGCCAAATCCCATGCT-3' (NM_001004). The cDNA products were amplified with PfuUltra Hotstart DNA polymerase (Stratagene, CA) by 33 cycles (94˚C for 30 sec, 65˚C for 30 sec and 72˚C for 2 min), preceded by 2 min at 94˚C and followed by 7 min at 72˚C using reverse (as above) and forward primers with the following sequences: 5'-ATGC CCAGGGAAGACAGGGCG-3' (P0), 5'-ATGGCCTCTGTC TCCGAGCTCG-3' (P1) and 5'-ATGCGCTACGTCGCCTC CTACC-3' (P2). The purified PCR products, amplified with primers containing the *Eco*RI site, were inserted into pGEX-2T vector (Amersham Pharmacia Biothech, Freiburg) and sequenced. Single colonies of pGEX-P0, pGEX-P1, pGEX-P2 and pGEX transformed DH5α *E. coli* (Invitrogen, Italy) were induced with IPTG (1 mM) and GST (glutathione S-trasferase) and GST-tagged proteins were purified using GSTrap FF (Amersham, Bioscences Corp., USA). Proteins concentration was determined by Bradford protein assay (Bio-Rad, USA) (30). Proteins were visualized by Coomassie blue staining.

Immunofluorescence staining and flow cytometric determination of the C-22 P0 epitope on pharynx cancer cells. Indirect immunofluorescence was carried out on fixed and native cells at 25˚C and at 4˚C, respectively, using MAb 2B2 or MOPC-21 (1 μg/ml) (27,31). To stain native cells, FaDu cells were grown for 48 h with or without 10% serum. Cells were detached by incubation with 0.02% EDTA in PBS. For flow cytometric assessment, MAb 2B2-specific mean fluorescent intensity (MFI) was determined after subtraction of that obtained with the MOPC-21 in triplicate experiments. Propidium iodide staining (≤5% cells) was used to determine cell membrane integrity.

Tissues and sera. Tissues and sera were obtained according to the ethical guidelines of the Istituto Nazionale dei Tumori 'G. Pascale', Naples. Samples were obtained with patients' informed consent. Fifty-seven invasive carcinoma tissues

Table I. Expression of the epitope recognized by MAb 2B2 on carcinoma cell lines by ELISA.

Cell line	MAb 2B2 epitope expression
HNSCC	
FaDu	+++[a]
CAL 27	+++
CAL 33	++
SCC-15	++
Breast carcinoma	
MCF-7	+++
Lung carcinoma	
A549	+++
Mesothelioma	
Epithelioid	++
Sarcomatosus	+++
Biphasic	+++
Ovary carcinoma	
OVCAR	++

[a]Intensity of immunoreactivity has been determined according to the optical density at 492 nm: -, 0-0.2; +, ≥0.21≤0.5; ++, ≥0.51≤1; +++, ≥1.1≤2.5.

originated from the tongue (n=21), gum (n=1), floor of the mouth (n=7), palate (n=6), lip mucosa (n=2), mandibular mucosa (n=8), nose mucosa (n=2), pharynx (n=1), larynx (n=6) and parotid gland (n=3), included 53 squamous cell carcinomas, 2 mucoepidermoid and 2 undifferentiated carcinomas. Carcinomas were graded according to WHO classification as G1 (n=9), G2 (n=28) and G3 (n=20). Normal epithelium adjacent to carcinomas (n=43) was used for comparison, while dysplastic lesions and *in situ* carcinomas were evaluated in 25 and 9 cases, respectively. Benign tissues included 11 papillomas, while acanthotic epithelium occurring in inflammatory lesions was informative in 8 cases. Serum available from 40 out of the 57 patients was collected prior to therapy and compared to 39 healthy donor sera.

Immunohistochemical analysis. Expression of C-22 P0 epitope on tissues was determined by immunoperoxidase staining by incubation with MAb 2B2 or MOPC-21 (1 μg/ml) for 30 min at room temperature (31,32). Epithelium staining intensity was semiquantitatively classified as negative (-), weakly positive (± and +) or overexpressed (2+ and 3+).

Immunoblotting. Purified P-GST and GST proteins (0.5-2.5 μg/lane) as well as cell line extracts (50 μg/lane) were separated by SDS-PAGE (33). Membranes were incubated with MAb 2B2, MOPC-21 or human sera at the above concentrations. The 1:100 serum dilution was chosen since it was the highest serum concentration lacking background reactivity. The immunocomplexes were visualized as previously described (33).

Statistical analysis. Statistical associations were considered significant at p-values ≤0.05 using Fisher's exact test or Student's t-test.

Results

Production of a monoclonal antibody (MAb 2B2) that recognizes a conserved carboxyl-terminal epitope of the ribosomal P0, P1 and P2 proteins. After somatic hybridation, the hybridoma clone 2B2 was selected for the production of an antibody reacting in ELISA with the immunogen as well as with other carcinoma cell lines (Table I). A further screening carried out by immunohistochemistry indicated that MAb 2B2 recognized an antigen overexpressed by five human tongue carcinomas compared to the adjacent normal mucosa (data not shown). Proteomic analysis identified this antigen as the P0 60S acidic ribosomal protein (Swiss-Prot Entry P05388), characterized by the high homology in the last 22 amino acids with the other two members of the family designated P1 and P2 (C-22 P0 epitope, Fig. 1A). MAb 2B2 immunoreactivity with all three purified recombinant fusion GST-tag P proteins, migrating with an apparent molecular weight of 65 kDa (P0), 46 kDa (P1) and 44 KDa (P2) and its lack of reactivity with the GST protein indicated that MAb 2B2 identified a common P proteins epitope (Fig. 1B and C). Specificity of MAb 2B2 for the 22 amino acid residue carboxyl-terminal epitope (C-22 P0 epitope) was determined by ELISA. MAb 2B2 strongly reacted with the C-22 but not with the control peptide thus indicating MAb 2B2 recognition of the C-22 P0 epitope (Fig. 1D).

Expression and subcellular localization of the C-22 P0 epitope on head and neck cancer cell lines. The expression and subcellular localization of the C-22 P0 epitope on head and neck cancer cell lines was determined by Western blot and immunofluorescence analysis, respectively. MAb 2B2 detected by Western blotting, on head and neck cancer cell lines, two strong immunoreactive proteins of 38 and 19 kDa, consistent with the molecular weight of P0 and P1 proteins respectively and a weak immunoreactive product of 17 kDa consistent with that of the P2 protein (Fig. 2A). No reactivity was observed with the MOPC-21 control antibody (data not shown). Fig. 2B, shows a strong MAb 2B2 cytoplasmic immunostaining of all cell lines analyzed after permeabilization as well as the C-22 P0 epitope cell membrane expression on native cancer cells. No reactivity was observed with the MOPC-21 control antibody.

Overexpression of the C-22 P0 epitope in pathological lesions arising in the head and neck stratified epithelium. Expression of the C-22 P0 epitope in 57 invasive carcinomas was detected by immunohistochemistry and independently evaluated according to criteria of both relative intensity and homogeneity of epithelial cell staining. Expression in invasive carcinomas was compared with that of adjacent normal mucosa, acanthotic epithelium, benign tumors, dysplastic lesions and *in situ*

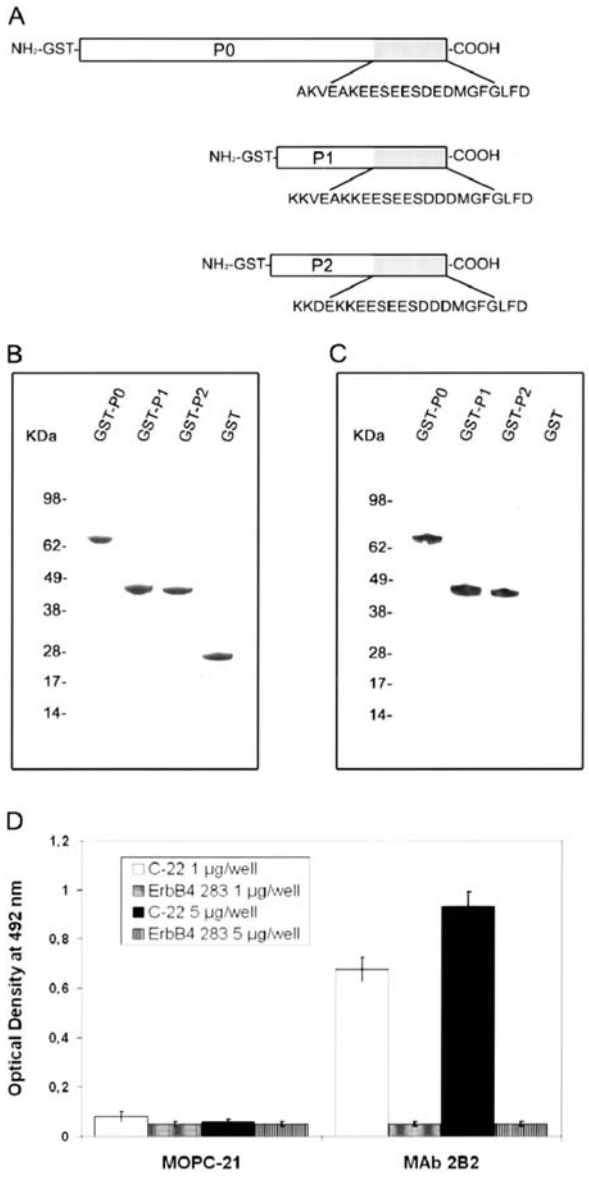

Figure 1. MAb 2B2 recognition of the C-22 P0 epitope. A, Schematic diagram of the homologous sequence of the C-22 P0 epitope on ribosomal P proteins. B, Coomassie blue staining showing purity of the human recombinant GST-tag P proteins and GST protein as indicated. C, Western blot analysis showing MAb 2B2 immunoreactivity with the purified recombinant GST-tag P proteins as indicated. D, Enzyme-linked immunosorbent assay showing immunoreactivity of MAb 2B2 with the C-22 peptide (C-22 P0 epitope) and not with a control peptide (ErbB4 283) at different concentrations as indicated.

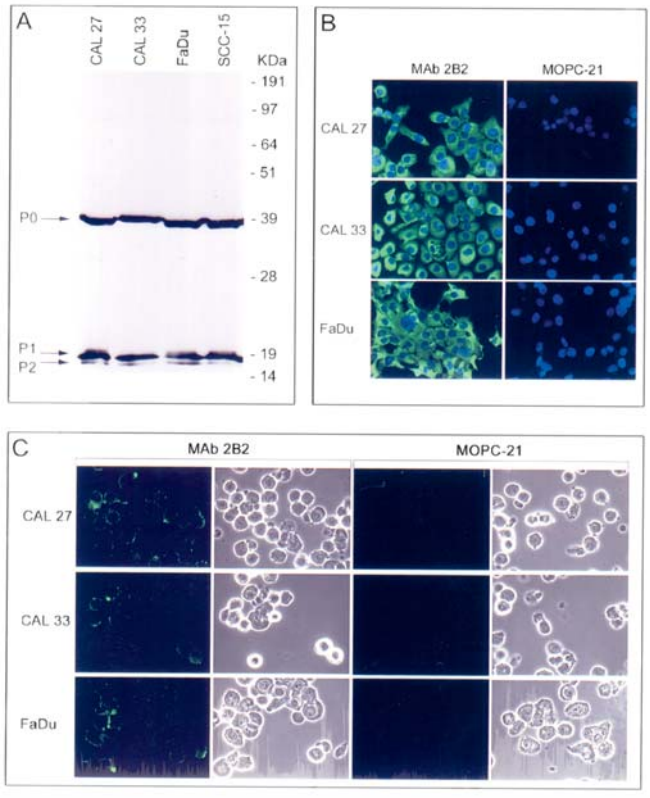

Figure 2. Expression and subcellular localization of the C-22 P0 epitope on head and neck squamous cancer cells. A, Western blot analysis showing MAb 2B2 immunoreactivity with tongue and pharynx cancer cells. B and C, Indirect immunofluorescence showing immunoreactivity of MAb 2B2 with fixed and permeabilized (B) or native (C), tongue and pharynx cancer cells as indicated. Detection of the C-22 P0 epitope on the cytoplasm (B) and membrane (C) of squamous cancer cells. Nuclei of permeabilized cells were counterstained using Hoechst 33342 (blue) (B). Cell morphology was determined by phase-contrast microscopy (C). MOPC-21 was used as negative control antibody. I.A.S. version 007 000 Deconvolution 2D software (Delta Sistemi, Italia) was used to deconvolve z series images of stained native cells (C).

carcinomas. Normal epithelium showed a weak and heterogeneous expression (Fig. 3Aa, Ba and f). Conversely, homogeneous overexpression was confined to benign tumors, dysplastic lesions and carcinomas, as well as to the acanthotic epithelium (Table II and Fig. 3).

A high (n=9) or moderate (n=28) degree of differentiation of the carcinomas gave the opportunity to determine the C-22 P0 epitope localization in defined cell layers of invasive tumors. While in adjacent normal epithelium the C-22 P0 epitope expression was limited to the basal cell layer (Fig. 3), overexpression in acanthotic epithelium, in dysplastic lesions and *in situ* carcinomas was observed both in the basal and in the intermediate cell layers (Fig. 3). Conversely, in 11 benign tumors, 7 showed overexpression in the basal and intermediate cell layers, while 4 showed overexpression limited to the intermediate cell layer (Fig. 3). A restricted intermediate cell layer overexpression was also observed in 7/9 and 4/28 well and moderate differentiated tumors, respectively (G1 vs G2 $p=8.9x10^{-4}$) (Fig. 3). On the other hand, overexpression in the basal and intermediate cell layers was observed in 1/9 and 23/28 for G1 and G2 carcinomas, respectively (G1 vs G2 $p=2.5x10^{-4}$). All undifferentiated tumors showed overexpression throughout all cell layers. MAb 2B2 showed also vessel immunostaining. No reactivity was observed with MOPC-21 (data not shown).

Overall, the C-22 P0 epitope was weakly expressed in normal epithelium. By comparison, among the 57 invasive carcinomas, the epitope was overexpressed, independently by the cell layer localization in 55 tumors, while among 25 dysplastic lesions it was overexpressed in 23 and in all *in situ* carcinomas (Table II). The C-22 P0 epitope overexpression was significantly associated with all pathological lesions analyzed arising in the head and neck stratified epithelium (Table II). However, overexpression in invasive carcinomas, was independent on the differentiation grade or nodal status (Table II).

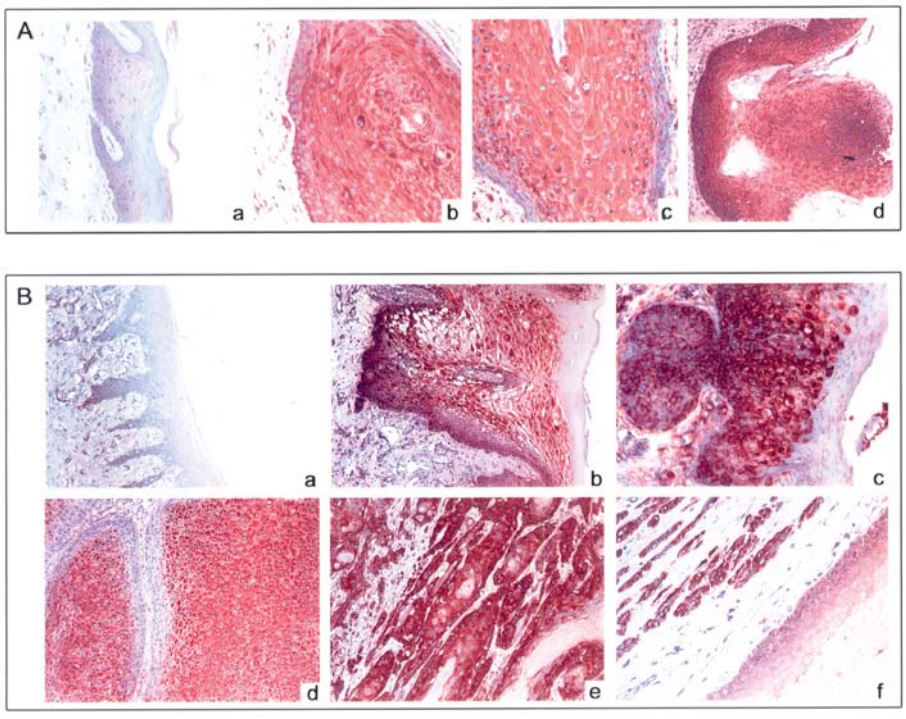

Figure 3. Ribosomal P0 protein overexpression in pathological lesions arising in the head and neck stratified epithelium. Immunohistochemical detection. A, Normal oral mucosa showing a weak immunoreactivity of the basal cell layer (a) [original magnification (o.m.) x200]; strong immunoreactivity throughout the acanthotic epithelium of a laryngeal nodule (Reinke's edema) (b) (o.m. x200); weak immunoreactivity of the basal cell layer similar to that in normal mucosa as opposite to the strong immunostaining of the intermediate cell layer in a squamous papilloma of the oral cavity (c) (o.m. x200), strong immunoreactivity throughout all the epithelium cell layers of a papilloma of the epiglottis (d) (o.m x100). B, Normal oral mucosa showing a weak immunoreactivity of the basal layer (a) (o.m. x100); low grade oral intraepithelial neoplasia with cellular acanthosis, exhibiting a strong immunostaining of all epithelium cell layers (b) (o.m. x200); high grade oral intraepithelial neoplasia/*in situ* carcinoma revealing a strong immunostaining of all epithelium cell layers (c) (o.m. x200); weak immunostaining of the basal cell layer as opposite to a strong immunostaining of the intermediate cell layer in a well differentiated squamous cell carcinoma (d) (o.m. x100); strong immunoreactivity throughout all cell layers in a poor differentiated squamous cell carcinoma (e) (o.m. x200); comparison of the C-22 P0 epitope expression in normal oral mucosa and a poorly differentiated oral squamous cell carcinoma (f) (o.m. x100).

Table II. Overexpression of the C-22 P0 epitope in HNSCC.

	C-22 P0 epitope overexpression	P[a]
Normal epithelium (n=43)	0	
Acanthotic epithelium in inflammatory lesion (n=8)	8	$1.5x10^{-9}$
Benign tumors (n=11)	11	$1x10^{-11}$
Dysplasia (n=25)	23	$3.7x10^{-16}$
In situ carcinoma (n=9)	9	$2.7x10^{-10}$
Invasive carcinoma (n=57)	55	$2.5x10^{-26}$
G1 (n=9)	8	
G2 (n=28)	27	
G3 (n=20)	20	
N0 (n=27)	25	
N+ (n=30)	30	

[a]Pathological tissue vs normal epithelium, two-tailed analysis.

Immunoreactivity of HNSCC patient sera with ribosomal P proteins. Employing the GST-fusion P proteins and the GST protein as negative control, serum from 40 of the 57 HNSCC patients and 39 from normal donors, was subjected to qualitative immunoblot analysis. Representative experiments are shown in Fig. 4A. None of the healthy donor sera showed reactivity with P proteins. The lack of reactivity with increasing concentration of P proteins (≤2.5 μg/lane) ruled out a dose-dependent immunoreactivity. Seven out of the 40 sera reacted with the P0 protein. Immunity to P0 protein was significantly associated with malignancy (p=0.011) (Table III). Simultaneous reactivity of 4 patient sera with all P proteins indicated either recognition of the C-22 P0 epitope or simultaneous immune response to different P protein epitopes. Indeed, HNSCC patient sera, recognizing all P proteins (#49, 78, 84 and 62), showed by ELISA, strong immunoreactivity with the C-22 P0 epitope, thus indicating the presence of autoantibodies against this common carboxyl-terminal epitope (Fig. 4B). On the other hand, sera recognizing only the P0 protein (#34, 21 and 85) failed to react with the C-22 P0 epitope, thus indicating recognition of epitope/s different from the C-22. No reactivity with the C-22 peptide was found from healthy donor sera (Fig. 4B). It is noteworthy that specific anti-P proteins autoantibodies were restricted to the IgG class (data not shown).

Spontaneous immune response to P0 protein was found to correlate with lymph node status but was independent of its

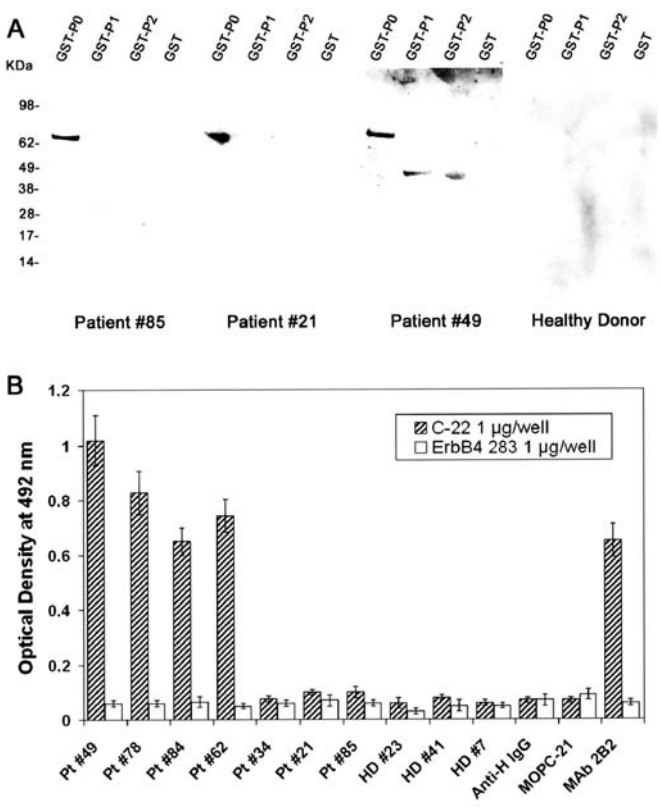

Figure 4. Detection of anti-P ribosomal antibodies in HNSCC patients. Immunoblot analysis of GST-tag P proteins and GST employing serum from HNSCC patients #85, 21 and 49 or healthy donor (A). Enzyme linked immunosorbent assay showing immunoreactivity of HNSCC patient (Pt. #) or healthy donor (HD #) sera with the C-22 P0 epitope. The ErbB4 283 peptide was used as control peptide. Anti-human IgG (Anti-H IgG) peroxidase-conjugated antibody alone was also included in the experiments. Experiments were repeated twice in duplicate. Column optical density values represent the mean ± standard deviation.

Table III. Immunoreactivity of HNSCC patient sera with ribosomal P proteins.

Serum origin	Nodal status	Serum reactivity with P0	Serum reactivity with P0+P1+P2
Healthy donors (n=39)		0	0
HNSCC (n=40)			
	N0 (n=19)	0	0
	N+ (n=21)	7 (p=0.011)[a] (p=0.0089)[b]	4[c]

[a]HNSCC patients vs healthy donors serum reactivity; [b]Ab+N+ (n=7) vs Ab-N+ (n=14) two-tailed analysis; [c]Four patient sera out of the 7 reacting with P0 protein showed simultaneous reactivity with P1 and P2 proteins.

overexpression in carcinomas. Seven of the 21 lymph node-positive patients revealed anti-P0 serum antibodies, while none of the 19 lymph node-negative patients showed anti-P protein autoantibodies (p=0.0089) (Table III). This result indicated a significant association of the anti-P0 ribosomal protein immune response with advanced disease stage.

Cellular stress increases the plasmamembrane expression of C-22 P0 epitope on pharynx cancer cells. Indirect immunofluorescence and flow cytometric analysis was employed to determine whether cellular stress induced by serum deprivation resulted in modification of the C-22 P0 epitope plasmamembrane expression on FaDu cells. Cells grown without serum showed a stronger membrane immunostaining than those cultured in serum (Fig. 5). The increased plasmamembrane expression of the C-22 P0 epitope, was independently confirmed by flow cytometric analysis. MFI of MAb 2B2 stained FaDu cells increased 3-fold upon serum deprivation (38±4 on unstressed cells vs 119±24 on stressed cells, p=0.0046). Conversely, no significant differences were found in the percentage of cells expressing the C-22 P0 epitope (54% ±8 on unstressed cells vs 72% ±18 on stressed cells) (Fig. 5).

Discussion

Immunity to self antigens has been previously reported in cancer patients and only sporadically in HNSCC patients (31-35). Nevertheless, we report on a spontaneous humoral immune response to P0 ribosomal protein associated with metastatic disease in HNSCC patients. To our knowledge, this is the first evidence of a spontaneous immune response to ribosomal P0 protein in cancer patients. Furthermore, the involvement of T helper cells was substantiated by the evidence that P0 protein autoantibodies belong to the IgG isotype. Autoantibodies can be generated through exposure of intracellular proteins at the surface of apoptotic cells (36,37). In this regard, P0 protein during the apoptotic process is concentrated on the cell membrane (22,23,38,39). Autoantibodies to ribosomal P proteins are frequently elicited in LES patients and are considered as a part of a specific disease process (38,39). In this respect we demonstrated that cellular stress leads to an increase of the C-22 P0 epitope on pharynx cancer cell surface. In large tumors, cellular stress such as hypoxia or nutrient deprivation may precede catastrophic biological events leading to cell apoptosis or necrosis (40,41).

The evidence that spontaneous immunity to P0 protein is elicited in 7 out of 40 HNSCC patients is apparently conflicting with previous observations sustaining an immune response dysfunction in HNSCC patients (42). Although the same cohort of HNSCC patients exhibited a scarce immune response to ErbB receptors (31), immunity to P0 protein was clearly elicited in the same immunological scenario. Such an apparent discrepancy might be related to the strong immunogenicity of the P0 protein. Indeed, it has been demonstrated that P0 protein may act as an endothelial cell autoantigen in LES (43). We were able to demonstrate strong immunogenicity of the P0 protein following immunization of mice with live cancer cells, and isolate the hybridoma 2B2 that produced an antibody that strongly reacted with the C22 P0 epitope. P0 protein autoantibodies were associated to patients

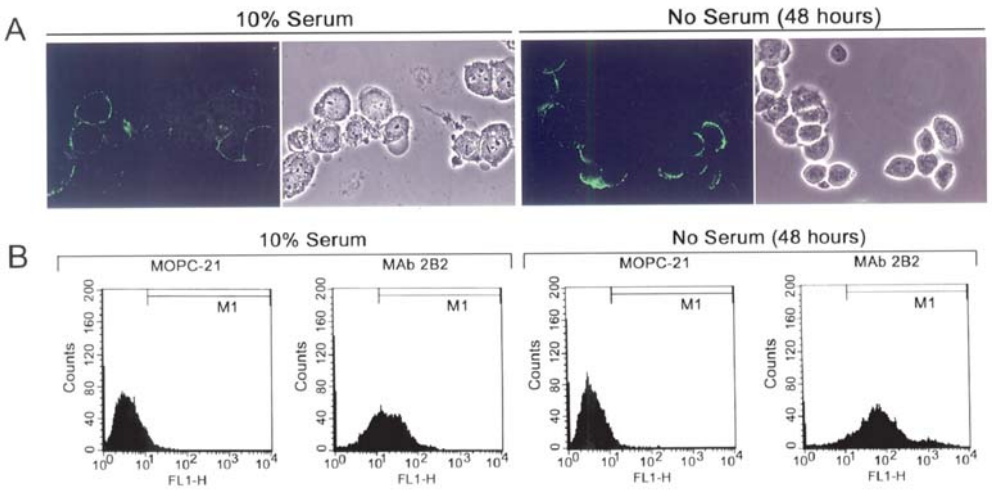

Figure 5. Increased detection of the C-22 P0 epitope following serum deprivation in plasmamembrane of pharynx squamous cancer cells. Indirect immunoflourescence (A) and flow cytometric assessment (B) indicating the C-22 P0 epitope surface expression on native pharynx squamous cancer cells after 48 h growth in the presence or absence of fetal bovine serum. Cells morphology was determined by phase-contrast microscopy (A). I.A.S. version 007 000 Deconvolution 2D software was used to deconvolve z series images of stained native cells. For flow cytometric determination, mean fluoresce intensity (MFI) was determined on the M1 cell labelled population.

with tumor-infiltrated but not with tumor-free lymph nodes. An increased frequency of autoantibodies associated with a late stage of tumor disease has been previously reported for p53 (44). Among the seven patients with spontaneous P0 immunity, five were classified as T4 (5/24) while one as T2 (1/11) and one as T3 (1/4). However, further studies need to be performed in a larger HNSCC patient cohort to establish whether the P0 autoantibodies might be considered a prognostic marker among patients with advanced stage disease.

The C-22 P0 epitope was immunodominant in HNSCC as found in LES patients (17-21). However, we demonstrated that at least in three patients, immunity to P0 protein was not restricted to the C-22 P0 epitope. Conversely, no immune response was observed to P1 or P2 proteins alone, thus indicating immunodominance of P0 protein. Although the P0 protein was overexpressed in several pathological lesions arising in the head and neck stratified epithelium including acanthosis, dysplasia, benign and malignant tumors, the immune response was not associated with P0 protein over-espression. It is noteworthy that overexpression in the acanthotic epithelium, in dysplastic lesions and *in situ* carcinomas, occurred in the basal and intermediate cell layers, while, it was restricted to the intermediate cell layer in well differentiated carcinomas as well as in some papillomas (4/11). Suprabasal squamous epithelium layer overexpression of tumor antigens as well as a Ki67 expression in premalignant and malignant lesions was previously reported (45-47). Although the basal cell layer is considered the proliferative compartment of normal stratified epithelium, the importance of the enhanced suprabasal proliferative activity as a consequence of cell replication outside the basal layer has been demonstrated (48). The restricted suprabasal P0 cell layer overexpression in selected tumors might suggest a potential cellular proliferation activity of the P0 in this epithelium layer.

Our findings might have clinical-pathological implications for HNSCC patients. The exposure of the C-22 P0 epitope on head and neck cancer cells as well as the induction of the spontaneous production of P0 autoantibodies indicate that the P0 protein might act *in vivo* as a spontaneous tumor immunological target. Furthermore, the presence of P0 autoantibodies linked to lymph node status might suggest the potential usefulness of the P0 elicited immune response as a prognostic marker among lymph node positive patients. Finally, the restricted C-22 P0 epitope overexpression on the suprabasal cell layer of preferred tumors might contribute to the understanding of biological behaviour of HNSCC.

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