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Simple Cytokeratins in the Serum of Patients with Lung Cancer: Relationship to Cell Death

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An important role in differentiation and proliferation has been demonstrated for the 20 cytokeratin (CK) polypeptides. The serum of 24 patients with biopsy-proven non-small cell lung cancer (NSCLC) and a similar number of controls was examined for evidence of CK8 and CK18. Using enzyme-linked immunosorbent assay (ELISA), all the control sera were negative, but 9 of the 24 patients were positive (mean 2.62 ng/ml; range 1.4–5.8; $P = 0.0036$). Western blotting confirmed the results of the ELISA in all cases, and indicated full size CK polypeptides. Advanced stage disease patients were more likely to be seropositive ($P = 0.00024$). Biopsy specimens showed CK8 expression in all 24 cases by immunochemistry and CK18 in 22 cases. This is the first study to demonstrate that a subgroup of NSCLC patients have intact CK8 and CK18 peptides in their serum, and their detection may correlate with advanced disease.
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INTRODUCTION

CYTOKERATIN (CK) POLYPEPTIDE-CONTAINING intermediate filaments are the structural unit of epithelial cells, and are found in the malignant tumours derived from them [1]. There are a total of 20 CK polypeptides, sub-divided into two equal families based on a combination of biochemical and molecular biologicial properties—the type I (acidic) and type II (basic) CK—and CK intermediate filaments are made up of equimolar quantities of each family [2–4].

The simplest pattern of CK intermediate filament expression

comprises CK8 and CK18, and is the first detected CK intermediate filament in embryonic lung [5]. As the adult lung develops, both the tracheobronchial epithelium and alveolar cells express a more complex pattern, but continue to express CK8 and CK18 [5, 6]. Similarly it has been shown by our group and other workers that all non-small cell lung cancer (NSCLC) tumours express CK8 and the majority CK18 [6–8].

Autoantibodies have been found to intermediate filaments in patients with a variety of disease processes, including lung cancer [9], and are presumed to result from cell destruction and liberation of cytoplasmic intermediate filaments into an environment where they can be presented to the immune system. However, few have demonstrated these to have diagnostic or prognostic value, with the exception of preliminary reports in non-malignant states associated with increased cell proliferation [10, 11].

The presence of CK polypeptides or degradation products in the serum of diseased patients may be assumed to be an essential prerequisite for autoantibody generation. Serum tissue polypeptide antigen (TPA), considered to be a product of degradation of CK8, CK18 and CK19 [12, 13], has been evaluated as a serological marker in patients with lung cancer. Limited specificity and clinical correlations have been shown [14, 15]. One study has demonstrated the presence of elevated levels of CK8 and degraded fragments of this polypeptide in patients with colonic and pancreatic tumours [16]. A further study in lung cancer patients, using serological detection of fragments of CK19, has indicated a possible prognostic role for this assay [17]. In this report, data are presented showing the presence of intact CK intermediate filaments in the serum of patients with NSCLC, but not in normal controls. A correlation has been found with clinico pathological stage, and preliminary data from *in vitro* studies suggest an association with death of malignant cells.

MATERIALS AND METHODS

Clinical data

24 lung cancer patients were selected from new referrals to the Cardiothoracic Centre (Liverpool, U.K.) or the Clatterbridge Centre for Oncology (Wirral, U.K.). The patients were staged in all cases by a chest radiograph, serum biochemistry and haematology. The serum samples were all taken from patients prior to any treatment. In 17 cases computer tomography (CT) scans were available, and in 1 patient thoracotomy had been performed.

Biopsy material

Formalin-fixed, paraffin-embedded biopsy samples were obtained from the 23 patients with lung cancer. Serial sections were cut at 4 μm with a Reichert 2030 rotary microtome (Reichert-Jung, Nuffloch, Germany), floated out on water in a bath heated to 48°C and picked up on to slides, which were then dried overnight in a 37°C incubator.

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Immunocytochemistry

The avidin–biotin–peroxidase technique was used to stain sectioned biopsy material and fixed cell monolayers [18]. Sections or fixed monolayers were incubated with primary monoclonal anti-CK antibodies M20 (Sigma, Dorset, U.K.) to CK8, or BA 17.2 (Sigma) to CK18 at dilutions of 1:200 and 1:100, respectively, prior to incubation with a biotinylated rabbit secondary antibody (BRAM, Dako, Copenhagen, Denmark) at a dilution of 1:300 and then an avidin–biotin complex (ABC) horseradish peroxidase (HRP) conjugate (Dako) at a dilution of 1:50. The sections were reacted with 0.05% diaminobenzidene (DAB) (Sigma) in 0.01% hydrogen peroxide in phosphate-citrate buffer, and the cell nuclei were counterstained with haematoxylin.

Preparation of serum samples

Serum was prepared from 20 normal, healthy control subjects and 23 patients with histologically proven, untreated lung cancer.

Enzyme-linked immunosorbent assay (ELISA)

A CK ELISA kit (Biogenesis, Franklin, U.S.A.) was used to examine the serum samples for the presence of CK8 and CK18. CK standards and serum samples were added, in triplicate, to a 96-well plate, which had been precoated with a monoclonal antibody, then a HRP tracer was added to each well, and the plate incubated at 37°C for 2 h. The wells were then aspirated and washed with phosphate-buffered saline (PBS) containing 0.2% Tween 20 a total of three times, followed by one wash with phosphate-citrate buffer. Immunoreactions were visualised by the addition of OPD substrate (10 mg of *O*-phenylene-diamine dihydrochloride in 25 ml of phosphate-citrate buffer), and terminated after 30 min by the addition of 1 mol/l sulphuric acid. The optical density of each well was then determined at 490 nm using a MR7000 plate reader (Dynatech, Liverpool, U.K.).

Western blotting

Samples of serum, conditioned medium, A549 cell lysates (Courtesy of P. Browning, CRC Department of Cell Biology, Liverpool, U.K.) and prestained low molecular weight markers (Bio-Rad, California, U.S.A.), were resolved by one-dimensional SDS–PAGE on 10–15% gradient gels using a Phast System apparatus (Pharmacia, Bjorkgatan, Sweden). Gels were then diffusion blotted at 70°C on to nitrocellulose membranes [19], and either stained with Pelikan Indian ink to check protein transfer [20], or subjected to western blotting [21]. Briefly, blots were incubated with primary monoclonal anti-CK antibodies M20 to CK8, or BA17.2 to CK18 at dilutions of 1:200 and 1:100, respectively, prior to incubation with BRAM at a dilution of 1:1000, and then an ABC–HRP conjugate at a dilution of 1:50. Immunoreactions were visualised using 0.05% DAB (w/v) containing 0.01% (v/v) hydrogen peroxide in PBS, and the stained nitrocellulose membranes were then scanned using a Schimadzu CS9000 densitometer (Schimadzu, Tokyo, Japan).

Cell culture

The human lung adenocarcinoma cell line, CaLu3 [22], was cultured in Dulbecco's modification of Eagle's minimum essential medium (DMEM) supplemented with 10% newborn calf serum (NCS) (Gibco, Gaithersburg, U.S.A.), containing 50 μg/ml gentamycin (Sigma). Confluent CaLu3 monolayers were maintained in serum-free conditions for 7 days in either 5% CO_2 in air or under anoxic conditions. Tissue culture supernatant

was collected from both groups, centrifuged to remove debris and concentrated by ultrafiltration on a membrane with a molecular weight cut-off of 10 000 Da (Amicon, PM10, Beverley, U.S.A.). Adherent viable cell monolayers for immunocytochemistry were fixed in methanol at −20°C.

RESULTS

Of the 24 patients (19 male, 5 female) with biopsy-proven NSCLC studied, the mean age was 62 years (range 53–81). Routine histopathology grouped the cases into 18 squamous cell carcinomas, two adenocarcinomas, one large cell carcinoma and three cases classified as non-small cell carcinoma of unspecified type. The stages were stage II in 12 cases, IIIa in 4 cases, IIIb in 7 cases and IV in 1 case with hepatic metastases.

Immunocytochemistry for CK8 was positive in the biopsy material for all 24 cases, and positive for CK18 in 22 cases (Fig. 1). CK8 and CK18 were also demonstrated in viable CaLu3 monolayers.

CK8/18 ELISA was positive in 9 lung cancer patients sera, with a mean value of 2.62 ng/ml (range 1.4–5.8). All positive cases and 7 of the cases negative by ELISA were then analysed by western blotting using monospecific anti-CK antibodies raised against peptides 8 and 18. In the ELISA-positive cases, all 9 were positive for CK8, whilst 6 of the 9 were positive for CK18. Calculation of molecular weight of the immunoreactive bands gave values of 51 900 kD with anti-CK8 and 44 700 kD for anti-CK18. The densitometry results of the western blotting experiments were first calculated as a fraction of the A549 standard (mean 0.14, range 0–0.47) and then correlated with the quantities determined by ELISA. The correlation by Pearsons product moment is shown graphically in Fig. 2, and was significant with $r=0.87$ (95% confidence interval 0.65–0.95), $P < 0.0001$. All negative cases were confirmed by western blotting analysis with both antibodies. All of the 20 healthy controls were found to have less than 0.2 ng/ml of CK8 or CK18 in their sera. Equally, western blotting analysis of the control sera showed no reaction with CK8 or CK18 antibodies.

Using a two-tailed Fisher's exact test, a significant correlation between the presence of serum CK8/18 in NSCLC patients versus controls ($P = 0.0036$) and NSCLC patients with advanced stages IIIb+IV versus II+IIIa ($P = 0.00024$) was found. There was no correlation between sex or histopathological

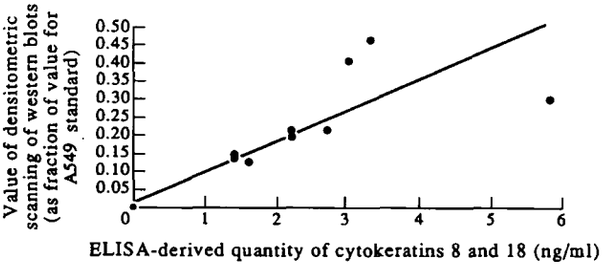

Fig. 2. Scatter plot of ELISA quantitation versus western blotting densitometry values for NSCLC patients serum cytokeratins 8 and 18.

sub-type of carcinoma, nor with the presence or level of serum cytokeratin polypeptide. However, the 1 case with liver metastases did have double the mean value of serum CK of the malignant cases.

TPA at a concentration of 2.85 ng/ml was used in both ELISA and western blotting experiments to determine whether reactivity in these systems could be achieved. In the ELISA, the appropriate quantity of TPA was detected, but no reaction was observed in the western blotting experiments with the antibodies used.

Lysates of the cell line A549 and conditioned serum-free medium from autolytic CaLu3, induced by anoxia, gave values of >15 ng/ml. However conditioned serum-free medium from adherent and viable monolayers of CaLu3, grown under normal conditions, contained no measurable CK8/18. The results obtained by ELISA with the CaLu3 conditioned medium were confirmed by western blotting (Fig. 3).

DISCUSSION

This study demonstrates that undegraded simple CK, known cytoplasmic consitituents of epithelial tumours, are found in the serum of a subgroup of patients with NSCLC. The presence of CK polypeptides correlated significantly with advanced disease stage, and not with other clinicopathological data or tumour tissue expression.

ELISA was chosen for its ability to identify quantitative differences in serum CK levels. While all the control group and a proportion of the patients with NSCLC studied were negative

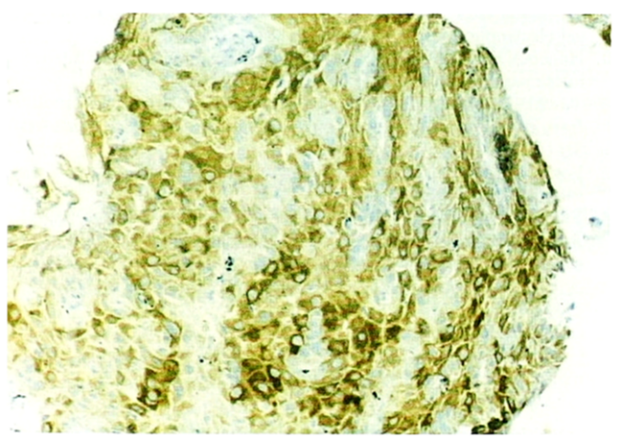

Fig. 1. Immunocytochemical demonstration of cytokeratin polypeptide 8 in the biopsy of a study patient with squamous cell carcinoma. Localisation was by chromogen DAB visualised as brown against a haematoxylin counterstain. Magnification × 200.

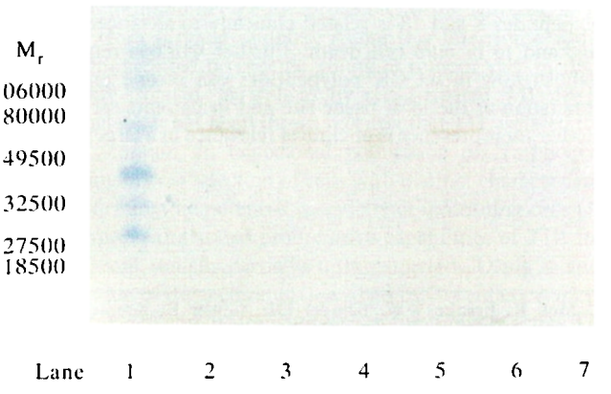

Fig. 3. Western blotting analysis of conditioned culture medium from CaLu3 using M20 anti-cytokeratin 8 probe. Lane 1, molecular weight standards 18 500 to 106 000; lane 2, A549-positive control 51 900; lane 3, CaLu3-conditioned medium (anoxia) 51 300; lane 4, CaLu3-conditioned medium (anoxia) 51 300; lane 5, A549-positive control 51 900; lane 6, CaLu3-conditioned medium (air), negative; lane 7, CaLu3-conditioned medium (air), negative.

by this assay, the patients who were serum-positive exhibited a wide range of concentrations from 1.4 to 5.8 ng/ml, and the 1 patient with extensive metastatic disease had a level over double the mean for the group. The quantities detected are low but comparable with that of another study of CK19 fragments in the sera of lung cancer patients [17]. Western blot analysis corroborated the ELISA data, and in addition, identified the molecular weight of the peptides measured by ELISA to be that of intact CK polypeptides, with the molecular weight of the immunoreactive bands being significantly greater than that of the potential degradation product of CK8, 18 and 19 TPA. Similarly, we have shown that the antibodies used in the western blotting experiments were not reactive with TPA, and a significant correlation between the values derived from densitometric scanning of the nitrocellulose membranes from western blotting experiments and the ELISA quantitation was observed.

It may be proposed that the CK polypeptides are released from the cell following autolysis, and previous studies, indicating the presence of serum autoantibodies to keratin, raised the possibility that intact or partially degraded CK peptides are released into the circulation at the time of cell death [9]. The solubility properties of intact CK polypeptides may favour their retention at the tumour site following necrosis, but partial degradation by enzymes may increase this solubility and could account for their presence in the serum [23], as degraded tail-less, rich polypeptides such as TPA. Thus, if intact CK polypeptides are detected in the serum of cancer patients, an alternative hypothesis regarding their release is required.

One study in human breast carcinoma cell lines has shown intact CK can be found on the cell surface, and shed into tissue culture medium in viable monolayers, while no release is noted in normal breast epithelial cells *in vitro* [24]. This contrasts with the results of our experiments, where death was induced by anoxia in half of a culture population of CaLu3 immunocytochemically positive for CK8 and CK18. However, the presence of these CK was only demonstrated in medium supernatant of the autolytic cells, and not in supernatants from those maintained in air-enriched conditions. While these experiments have demonstrated that cell death is associated with CK polypeptide release, it is possible that some tumours possess the ability to shed CK filaments without the necessity for autolysis.

The conclusions of this study are that CK polypeptides 8 and 18 can be found in the serum of a subgroup of NSCLC patients but not in normal, healthy individuals. The presence of CK polypeptides 8 and 18 is related clinically to advanced disease stage and to *in vitro* cell death. Further work is required to establish how intact CK polypeptides can escape proteolytic degradation at the local tissue site and in patients serum, and whether their presence is of clinical relevance in a larger series.

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