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A protein of squamous keratinising epithelium from odontogenic keratocyst fluid

J. Southgate, J.T. Whicher, J.D. Davies, D.St.J. O'Reilly, and R.W. Matthews

Departments of Chemical Pathology, Pathology, and Dental Medicine at the Bristol Royal Infirmary and University of Bristol, Bristol

Summary. Fluid from odontogenic keratocysts was analysed. The major protein fraction with a mobility anodal to albumin on electrophoresis was shown not to be albumin or pre-albumin but a non-serum protein. Using an antiserum to keratocyst fluid absorbed with human serum, non-serum components of the odontogenic keratocyst fluid were localised in squamous keratinising epithelia, principally in the upper layers. The same antiserum showed immunolocalisation in squamous cell carcinoma of skin and cervix. No localisation was seen in normal non-squamous epithelia, in liver, stomach and colon or in non-keratinising squamous epithelial cysts of the jaw.

Key words: Odontogenic keratocyst – Squamous epithelial antigen – Anodal protein – Immunoperoxidase technique

Odontogenic keratocysts differ in their origin, behaviour, and protein content from non-keratinising dental cysts (Toller 1970; Toller and Holborrow 1969; Forssell et al. 1974; Browne 1975, 1976; Ylipaavalniemi 1978). Fluid from such cysts is characteristic in appearance being cloudy and pale yellow in colour. In particular the fluid contains a lower concentration of soluble protein and may contain a major protein fraction with pre-albumin mobility on electrophoresis. Toller found this "prealbumin" band in seven of seventeen odontogenic keratocysts examined. The band was not seen in a series of seventy-one non-keratinising dental cysts (Toller 1970). The presence of the band has been suggested to be of value in the diagnosis of the keratocysts, a matter of some clinical importance, as such cysts, occurring in young people, show infiltrative properties and a tendency to recur after surgical removal. In this study the protein previously identified as a "pre-

Offprint requests to: J.T. Whicher, Department of Chemical Pathology, Bristol Royal Infirmary, Bristol, BS2 8HW, UK

albumin" (Toller 1970) has been further characterised. The distribution of the protein in human tissues was studied by means of immunocytochemical examination of a variety of normal and pathological tissue sections.

Materials and methods

Odontogenic keratocyst fluid. Fluid was aspirated from seven odontogenic keratocysts without blood contamination and stored frozen at −20° C. The cysts were confirmed to be odontogenic keratocysts by histological examination after subsequent surgical excision. Each of these cysts yielded about 1 ml of turbid fluid which on electrophoresis after centrifugation revealed the characteristic appearance of odontogenic keratocyst fluid including, in four of the cysts, the fast moving prealbumin band (Toller 1970). The bulk of this fluid from the four cysts containing the fast moving "pre-albumin" band was pooled.

Antiserum to odontogenic keratocyst fluid. Antiserum to pooled keratocyst fluid was raised in a chincilla rabbit using complete Freund's adjuvant. 50 µl of keratocyst fluid, protein content (15 g/l) was mixed with 1 ml of normal saline and emulsified with 1 ml of complete Freund's adjuvant and injected subcutaneously at four sites on the rabbit. The procedure was repeated at monthly intervals. Blood was obtained from an ear vein 10 days after the fourth inoculation. The antiserum was tested against keratocyst fluid and human serum by crossed immunoelectrophoresis and was then absorbed against human serum bound to cyanogen bromide activated Sepharose 4B according to the method of Porath et al. 1967.

Tissue homogenates. Tissues were homogenised in phosphate buffered saline and digested with collagenase (Sigma clostridiopeptidase A: E.C. No 3.4.24.3) for 23 h at 20° C in order to facilitate cellular disruption.

Double diffusion analysis of collagenase-treated digests of oral (buccal, palatal and tongue) mucosa against antiserum raised to keratocyst fluid and further absorbed with human serum was performed according to Ouchterlony in 1% agarose gels (Ouchterlony 1967). Well sizes were 4 mm diameter for the antibody and 2 mm diameter for the antigen. Tissue homogenates were used neat and in dilutions of 1:10, 1:50, 1:100 in electrophoresis buffer. Diffusion took place overnight at 30° C. The gels were then pressed, dried and stained with 2% Coomassie Blue.

Electrophoretic studies. Electrophoresis of keratocyst fluid was performed in 1% agarose gels using barbital buffer pH 8.6, ionic strength 0.02 with a field strength 8 V/cm and run for 30 min. Fixation was carried out in picric acid and staining was performed with 2% Amido Black. Crossed immunoelectrophoresis was carried out according to the method of Weeke (1973).

Antisera to whole human serum and prealbumin were obtained from Dakopatts A/S, Copenhagen, Denmark and used at a final concentration in the gel of 50 µl/ml. 2 µl samples of the keratocyst protein and anti-keratocyst protein at a concentration of 150 µl/ml of gel were used.

Immunoperoxidase localisation. 5 µm-thick paraffin sections were prepared from tissues fixed for 24 h to 48 h in fresh unbuffered formol-saline. Material was examined from normal human tissues: squamous epithelium from tongue, buccal and palatal mucosa, skin, vagina, cervix and oesophagus; stomach, liver and colon; odontogenic keratocysts, non-keratinising squamous cysts of the jaw and, squamous cell carcinomas of cervix and skin.

The antiserum to keratocyst protein was applied to untrypsinised tissue sections at a dilution of 1 in 50 with phosphate buffered saline. A standard peroxidase-antiperoxidase methodology was used (Davies et al. 1982). The antiserum was blocked in control sections after overnight incubation at 4° C with an aliquot of neat keratocyst fluid or human serum at dilutions up to 1 in 500. Further controls included omission of the primary antiserum, the use of non-immune rabbit serum, and the substitution of rabbit antiserum to an entirely unrelated antigen (Anti-transferrin, Dako, A/S Copenhagen).

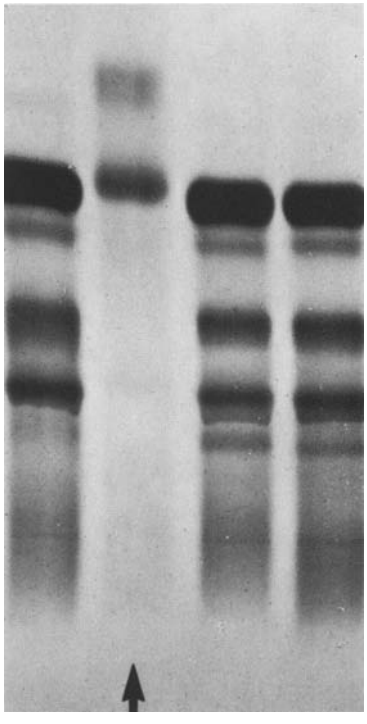

Fig. 1. Agarose electrophoresis of odontogenic keratocyst fluid (arrowed) compared with normal human sera. The anode is at the top of the figure

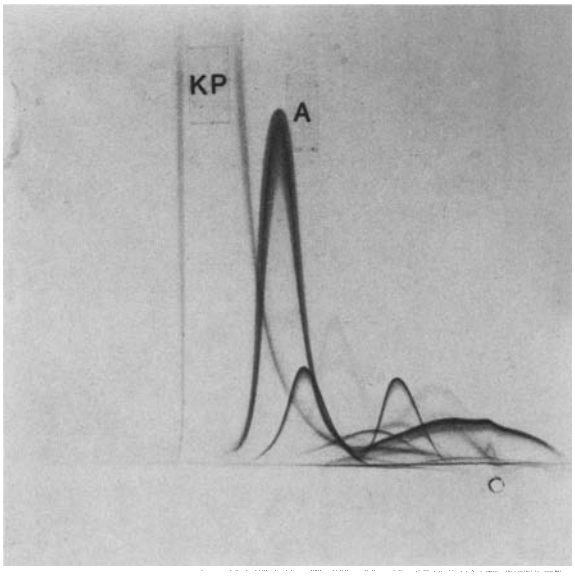

Fig. 2. Two dimensional electrophoresis (agarose) of odontogenic keratocyst fluid against an unabsorbed antiserum raised against pooled whole keratocyst fluid (anti k). 400 µl of antiserum and 2.5 µl of a 1:3 dilution of antigen in electrophoresis buffer were used. *A*, Albumin; *KP*, protein specific for odontogenic keratocyst

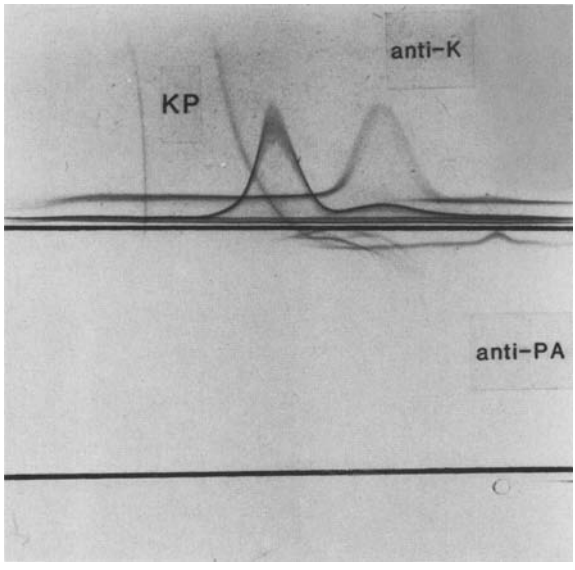

Fig. 3. Two dimensional agarose electrophoresis of pooled keratocyst fluid as shown in Fig. 2 but with an intermediate gel containing 200 µl of anti-pre albumin (anti-PA). Note that the KP (keratocyst protein) is not precipitated by the anti-prealbumin antiserum

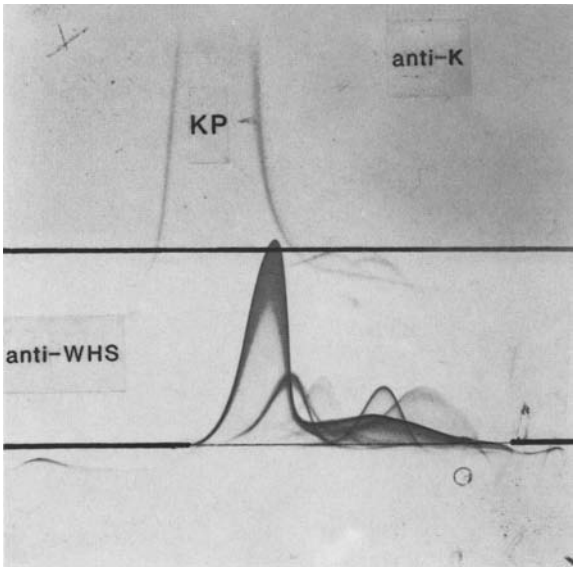

Fig. 4. Two dimensional agarose electrophoresis of pooled odontogenic keratocyst fluid as in Fig. 2 but with an intermediate gel containing 200 µl of anti-whole human serum (anti-WHS). The predominant protein KP and several minor proteins have passed through the intermediate gel without precipitation

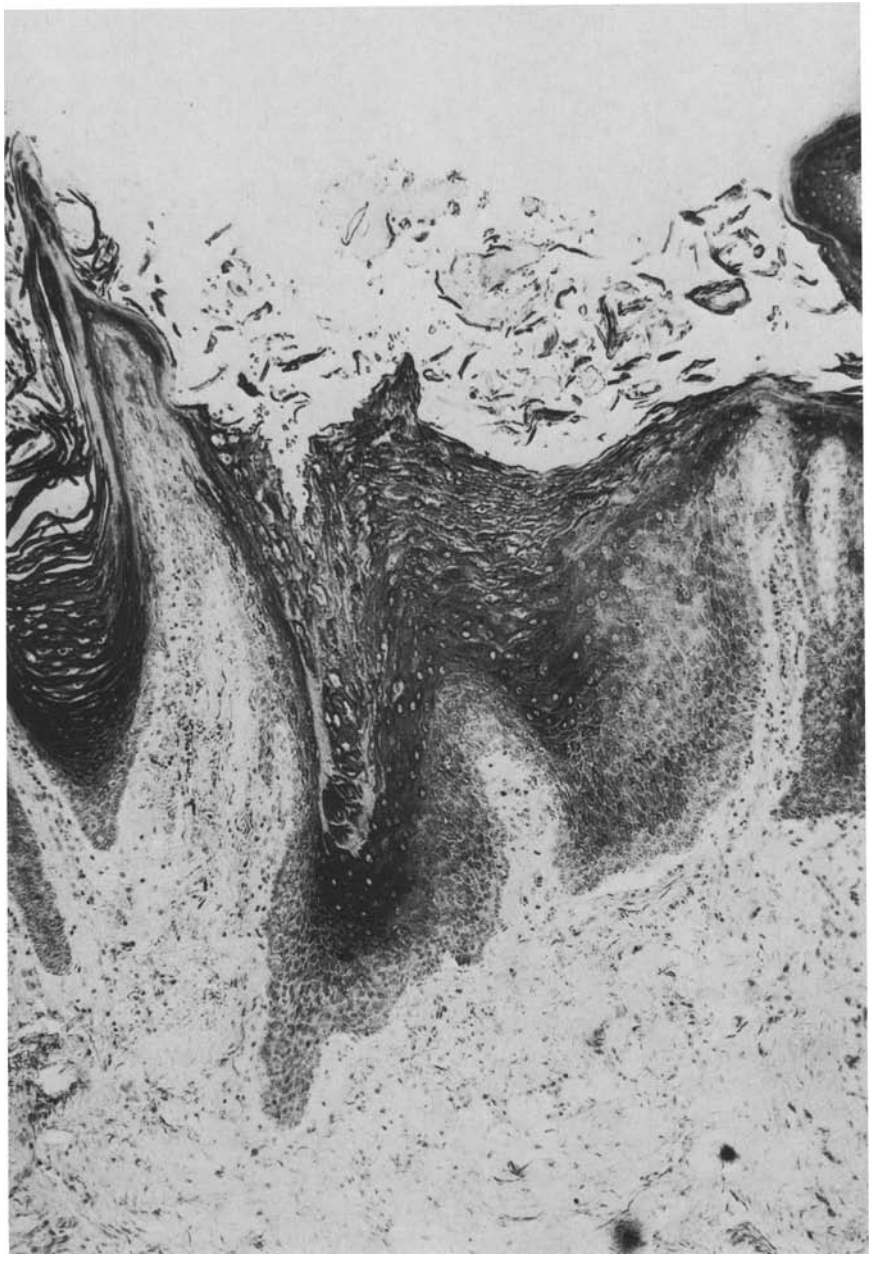

Fig. 5. Normal tongue mucosa immunostained with anti-keratocyst antiserum absorbed with normal human serum. Immunostaining is shown in the Malpighian and upper layers of the squamous epithelium. PAP and Haematoxylin counterstain. × 120

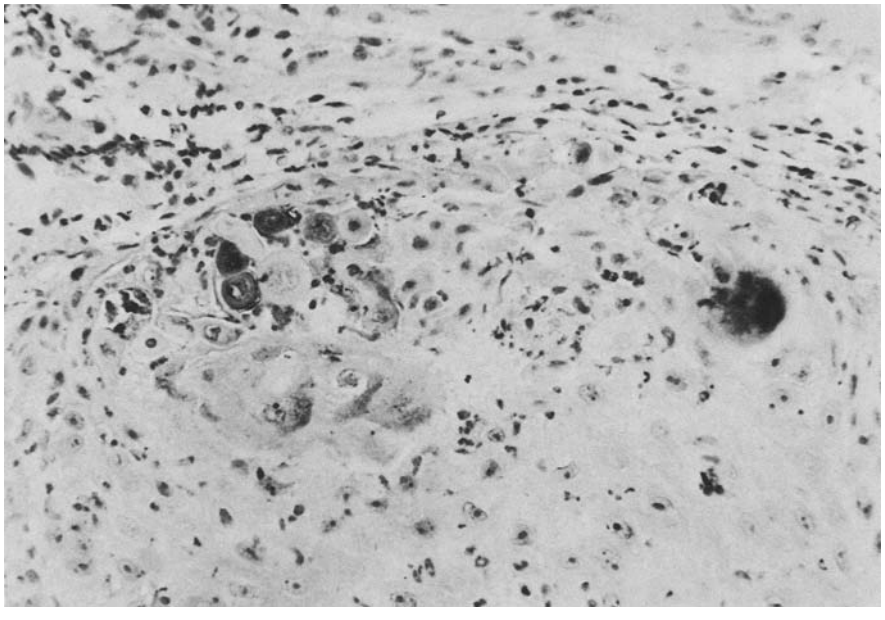

Fig. 6. Invasive squamous cell carcinoma of the tongue. Scattered individual squamous cells show cytoplasmic immunostaining with keratocyst antiserum after absorption with normal human serum. PAP and Haematoxylin counterstain. ×380

Results

Agarose electrophoresis of keratocyst fluid showed a prominent band anodal to albumin and running slightly in advance of the prealbumin bands of normal sera adjacent to it. Albumin was present and a further fainter band was seen in the β-globulin region (Fig. 1). Two dimensional electrophoresis of keratocyst fluid against unabsorbed antiserum raised to the keratocyst proteins showed that a number of proteins were present (Fig. 2), including albumin and a protein of more anodal electrophoretic mobility.

Further two dimensional analysis of cyst fluid with an intermediate gel containing anti-prealbumin showed that the major protein band anodal to albumin was not prealbumin as it passed through the intermediate gel to be precipitated by the antiserum to keratocyst fluid (Fig. 3). Similar studies incorporating anti-whole human serum (Fig. 4) showed one major and several minor non-serum proteins in the cyst fluid.

Ouchterlony double diffusion of absorbed antiserum to keratocyst fluid proteins against keratocyst fluid showed a single precipitin arc. No lines of precipitation were seen with human serum or homogenates or oral (buccal, palatal, tongue) mucosa. However, collagenase digests of oral mucosa (buccal, palatal and tongue) did produce a single precipitation line which showed identity with that produced by keratocyst fluid.

Immunoperoxidase staining of buccal, palatal and tongue (Fig. 5) epithelium, keratocysts, vaginal, cervical and cutaneous squamous epithelium

showed immuno-localisation in the Malphigian layer, and to a lesser degree in keratinising squamous epithelia in the superficial layers. This pattern of immunostaining was blocked by preliminary incubation of the antiserum with keratocyst fluid, but was not influenced by normal human or rabbit serum. No immunostaining was found when the primary antiserum was omitted, or after substitution of non-immune rabbit serum or rabbit antiserum to transferrin. No immunostaining was found in the epithelial components of normal oesophagus, liver, colon or stomach. In invasive keratinising squamous – cell carcinomas from skin and cervix a focally heavy immunostaining was found in keratinising cells of the invasive tumours (Fig. 6). No immunostaining was found in non-keratinising squamous cysts of the jaw.

Discussion

Using electrophoretic techniques we have shown that the major protein of dental keratocyst fluid, with an electrophoretic mobility anodal to albumin, is not prealbumin but a protein absent from normal human plasma.

An antiserum raised to odontogenic keratocyst fluid and absorbed by human serum reacts with one major and four minor non-plasma keratocyst proteins on crossed immunoelectrophoresis (Fig. 4). The major protein with which our antiserum reacts is probably the component of "prealbumin" electrophoretic mobility described by Toller (1970). A similar protein of fast electrophoretic mobility has been described by Kuusela et al. (1982). This protein had an approximate molecular weight of 50,000 daltons and an antiserum against it reacted, by immunofluorescence, with epithelial cells in the capsule of both keratinising and, in contrast to the finding with our protein, to non-keratinising dental cysts.

We examined the immunolocalisation of our absorbed antiserum in a wide variety of tissues. Immunoperoxidase staining of keratocyst epithelium, buccal and palatal mucosa, tongue, skin, cervical and vaginal epithelium showed localisation in the Malphigian and keratinising layers suggesting reactivity with a keratin-related protein. There was no localisation in non-keratinising cysts of the jaw, liver tissue or epithelia from oesophagus, colon or stomach. We were unable to demonstrate reactivity in Ouchterlony double diffusion between our antiserum and homogenates of oral keratinising mucosa. This was probably due to insolubility of the antigen. However, treatment of the mucosal samples with collagenase, but not with trypsin, pepsin or papain resulted in the release of solubilised antigen which was able to form immunoprecipates with the antiserum. This is of interest as keratocysts contain high levels of collagenase activity in their walls (Dornoff et al. 1972; Ylipaavalniemi 1978). This may result in partial digestion of structural protein components and to their release into cyst fluid and might contribute to the locally invasive properties of keratocysts.

The nature of the major protein of keratocyst fluid is unclear. Kuusela et al. (1982) showed it to be resistant to collagenase and this, together with its high antigenicity, suggest that it is not derived from collagen. By contrast

collagen peptides and gelatins are poorly antigenic. The localisation in the keratinising and Malphigian layers in our studies suggests a keratin related protein. However Kuusela et al. (1982) failed to show identity between antibodies to prekeratin and their keratocyst antiserum and also showed that antibodies to mature keratin did not react with keratocyst fluid. Such findings may indicate that the keratocyst protein is associated with keratin and keratin precursors, but is antigenically unrelated. Our observations extend those of Kuusela et al. in showing that the antigen is of widespread distribution in squamous keratinising epithelia. The immunostaining pattern of the keratocyst antigen differs from that described with involucrin (Watt 1983) in that the cell cytoplasm, rather than the membrane region was stained. However formalin fixation (Banks-Schlegal and Green 1981) diminished the antigenic properties of involucrin in the cell margin. The exact relationship between the keratocyst antigen and involucrin, which is a marker of terminal differentiation in keratinocytes, with a molecular weight estimated as 92,000 daltons and an isoelectric point of 4.5 (Rice and Green 1979), is uncertain.

The unique occurrence of a protein of fast anodal mobility in odontogenic keratocysts, the fluid content of which are low in protein derived from plasma, led us to examine the immunolocalisation of our antiserum in malignant tumours of squamous epithelia. Squamous cell carcinomas from both skin and cervix showed heavy immunostaining of the invasive cells. These findings are of some interest as the protein may have potential as a marker of tumours derived from squamous keratinising epithelium.

It is of interest that the protein was not detected in the fluid of all keratocysts examined but was localised in immunoperoxidase preparations of all preserved keratocysts examined albeit weakly in some cases. This may reflect the insensitivity of our electrophoretic method or perhaps the age of the cyst. Further work will be required to identify a much larger series of odontogenic keratocysts to determine whether the presence of a fast moving pre-albumin band on electrophoresis differentiates a true subclass of odontogenic keratocyst.

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