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Differentiating keratoacanthoma from squamous cell carcinoma by the use of apoptotic and cell adhesion markers

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Aims: Keratoacanthomas (KA) are well-differentiated squamoproliferative skin lesions that grow rapidly and regress spontaneously. In contrast, squamous cell carcinomas (SCC) can have variable differentiation, inexorably progress and on occasion metastasize. Distinguishing between KA and SCC using haematoxylin and eosin-stained sections from an initial biopsy can often be difficult. There is also some debate as to whether KA is simply a variety of well-differentiated SCC or a distinct entity.
Methods and results: Initial biopsy sections from 25 cases of SCC and 20 of KA were labelled with markers for both the initiation (the cytolytic receptor $P2X_7$) and end-stage (terminal deoxynucleotidyl transferase biotin–dUTP nick end labelling) of apoptosis, telomerase-associated protein (TP1) and the cell adhesion protein E-cadherin. As this was a retrospective study, the clinical outcome of each case was known. This resulted in a unique labelling pattern of each marker for SCC and KA, allowing a differential diagnosis between the two conditions. The simplest marker to use for this purpose was anti-$P2X_7$. Sections from five cases that were initially very difficult to diagnose were correctly identified as SCC using this method.
Conclusions: These results support the view that KA has a different pathogenesis and biochemistry from that of SCC, and is a distinct entity. Anti-$P2X_7$ labelling, using routine immunohistochemical techniques, provides a method for differentially diagnosing these conditions.

Keywords: apoptosis, keratoacanthoma, purinergic receptors, squamous cell carcinoma

Abbreviations: KA, keratoacanthoma; SCC, squamous cell carcinoma; TP, telomerase-associated protein; TUNEL, terminal deoxynucleotidyl transferase biotin–dUTP nick end labelling

Introduction

Keratoacanthoma (KA) and well-differentiated squamous cell carcinoma (SCC) are two cutaneous neoplasms that most often occur in sun-exposed sites of light-skinned persons. It may be difficult to distinguish KA from SCC both clinically and histologically, as the cytological features are similar in both tumours. Many of the criteria commonly used for the differential diagnosis of SCC and KA are not reliable.[1–3]

KA typically presents as a dome-filled lesion with a central crater filled with keratin. The epithelium is well-differentiated and no abnormal mitoses are seen. This condition is thought to represent a proliferation of the infundibular portion of hair follicles rather than the epidermis, although the infundibulum may itself be of epidermal origin. It grows rapidly for 4–6 weeks and then undergoes spontaneous regression over the following 4–6 weeks, ultimately leaving a slightly depressed annular scar. SCC has a similar histological appearance, develops more slowly and rarely spontaneously regresses.[4] It is important to distinguish between these tumours, as KAs are benign lesions that spontaneously regress or may be treated by excisional biopsy, whereas SCCs are malignant tumours that have the potential to metastasize and require radical surgery.

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The main diagnostic difficulty is distinguishing between KA and a low-grade SCC. KA typically is a very fast growing lesion, which may paradoxically outgrow an SCC clinically, but it reaches a growth plateau and will then regress. Architecturally, KA has a central keratin plug, which because of its endophytic growth becomes enclosed in a crater (laterally buttressed by normal epidermis). SCC can have a central keratin crater, though usually the keratin/epithelial interface is more irregular and keratin pearls more pronounced. The epidermis appears bland in KA, with abundant cytoplasm. Minimal mitotic figures and (importantly) no abnormal mitoses are seen. The epidermal/dermal interface in KA is bulbous and pushing, though in the stage of regression a heavy lymphocytic infiltrate may leave a piecemeal irregular interface. A genuine infiltrating interface indicates a SCC, so lymphatic and perineural spaces should be scrutinized. Occasionally it is very difficult to distiguish between SCC and KA based on a haematoxylin and eosin (H&E) stain.

Some workers believe that these neoplasms are part of the same disease entity and that KA represents a hyperplastic premalignant lesion within the SCC spectrum.[2,5,6] Others, however, contend that they are separate entities.[7–9] In an attempt to address this question we compared the expression of specific markers for early and late-stage apoptosis in both groups of lesions. In this study we used an antibody to the cytolytic calcium channel receptor $P2X_7$ to indicate the initiation of apoptosis, terminal deoxynucleotidyl transferase biotin–dUTP nick end labelling (TUNEL) to indicate that apoptosis had proceeded to completion, an antibody to telomerase-associated protein (TP1) to indicate the neoplastic/proliferative state of the cells and anti-E-cadherin to indicate the cell adhesion status of the cells.

Materials and methods

Serial unstained sections were cut from paraffin blocks of 20 cases of established KA and 25 cases of established SCC. Of the SCC cases, five had been very difficult to diagnose histologically on the initial biopsy. The cases were supplied by the Anatomical Pathology Department of The Royal Prince Alfred Hospital (RPAH), Sydney, Australia. Ethical approval has been granted by the Central Sydney Area Health Service and RPAH, ethical approval protocol number X04-0020.

Tissue was selected on the basis of a retrospective review of all cases of KA and SCC archived on the RPAH Anatomical Pathology database from the last 10 years. Cases which demonstrated tissue/epithelium of interest were selected by staff specialist pathologists from the Anatomical Pathology Department database. Another consideration was that there was sufficient material available in the tissue block for slide preparation and also acceptable quality of tissue, based on the H&E histological appearance of original sections. The case notes accompanying each case indicated that the diagnosis was made and verified by two pathologists. The diagnosis was made from the presenting lesion and microscopic examination of the excised tissue. The corresponding paraffin-embedded tissue blocks were retrieved from file and nine 5-μm sections of each were cut and mounted onto Superfrost slides.

IMMUNOHISTOCHEMISTRY

Formalin-fixed paraffin-embedded biopsies were used for this study. Approximately serial sections were cut from the paraffin tissue blocks and stained using a standard H&E protocol. Each section was immunolabelled as previously described.[10] For immunohistochemistry, each slide was de-waxed in two changes of fresh Histoclear for 15 min each and then rehydrated. All sections were immersed in 3% hydrogen peroxide for 5 min, washed and then incubated with the appropriate primary antibody ($P2X_7$ and hTP1) at a concentration of 0.25 μg/ml affinity-purified IgG in PBS for 30 min. Thereafter, slides were washed three times in PBS for 5 min each, followed by a 30-min incubation with LSAB secondary (Dako, Carpinteria, CA, USA). All slides were then washed in PBS for 5 min and visualized using a 0.05% solution of diaminobenzidine (DAB) for 5 min, washed, dried and mounted in Entellan mounting medium (Merck, Darmstadt, Germany). Preincubation with peptide epitopes at 5–10 μM completely blocked labelling in preabsorption controls. The sections destined for E-cadherin labelling were first incubated in Target Retrieval Solution (Dako) at 70°C for 50 min, in order to unmask the epitope. The sections were then labelled with monoclonal anti-E-cadherin (Zymed, San Francisco, CA, USA) and were treated with primary antibody at a concentration of 1 : 100 in PBS, for 30 min. The human $P2X_7$ subtype-specific antibody has been described previously,[11–14] as has the TP1[15] antibody. The antibody titres, defined as the reciprocal of the serum dilution resulting in an absorbance of 1.0 above background in the ELISA assay, were all in the range 75 000–95 000 compared with 200–250 for the preimmune samples. For TUNEL labelling, the Dermatacs Apoptosis Detection Kit (R&D systems, Minneapolis, MN, USA) was used according to the detailed protocol supplied with the kit.

LABELLING INTENSITY QUANTIFICATION

Actual levels of antigen were not quantified in this study, but rather relative differences in labelling intensity were quantified using a standardized protocol.[16,17] In short, sections to be compared were labelled at the same time, using a single protocol. A Leica DC 200 digital camera using Leica 'DC Image' capture software was mounted on a Leitz Diaplan research microscope. The microscope illumination was fixed at 5 V. The exposure compensation option of the camera was deactivated and resolution set at 1798 × 1438 dpi. These precautions ensured that all images could be validly compared. The images were saved as TIFF files, transferred to a Macintosh G4 computer and opened in NIH Image 1.6 (written by Wayne Rasband and available on the Internet at http://rsb.info.nih.gov/nih-image/). Using the freehand box selection tool, 10 random measurements of 100 μm^2 each were made for each E-cadherin-labelled slide at ×100 magnification, resulting in an '*n*' of 400. Background readings from blank areas adjacent to the selection were made in each case and subtracted from each raw datum. Average and SD values were then determined. Epithelial selection reproducibility was tested by outlining a single defined area of epithelium 10 times, resulting in a variation coefficient of only 1.0%. The Alternative Welch *t*-test (two-tailed *P*-value) was chosen for analysis of the data because it does not rely on the assumption that the sampled populations have equal SDs. The null hypothesis was that the two population means were equal.

Results

The 20 cases of KA and the 20 cases of SCC possessed the usual diagnostic histopathological features required to make the respective diagnoses, as described in the Introduction.

In five cases, however, it was difficult to differentiate between a diagnosis of KA or SCC based on the initial H&E-stained sections. The case notes always included a statement by one or more reporting pathologists favouring a diagnosis of KA, SCC or the difficulty of diagnosing either. One case, shown in Figure 9, was so difficult to diagnose differentially that the concluding diagnosis simply stated that the features of both KA and SCC were present. The figures show the features of a representative clear-cut case of KA, one of SCC and, lastly, the difficult case previously mentioned.

In lesions diagnosed as KA, apoptosis was initiated and proceeded to cell death. Labelling for all antibodies tested was restricted to the upper epidermis adjacent to the keratin layer. Anti-$P2X_7$, TP1 and anti-E-cadherin labelling was coextensive and strong in these areas. TUNEL, anti-$P2X_7$ and anti-TP1-positive cells were found in the epidermis and close to the keratin layer. Cell adhesion, as measured by anti-E-cadherin, was found to be normal throughout the affected area. The labelling intensity of each antibody was similar in each case but the position and extent of the labelling varied.

In proven SCC lesions, however, anti-$P2X_7$ and TP1 distribution were again coextensive but extended from the surface of the epidermis to cell clusters near the deep edge of the tumour adjacent to the dermis. TUNEL labelling was negative, indicating that apoptosis, while it was initiated, did not proceed to cell death. E-cadherin labelling was reduced 3.1-fold ($P < 0.0001$) in SCC compared with that of KA, suggesting an increased propensity for cell migration. SCC was therefore characterized by intact neoplastic cells that were not destroyed by apoptosis. The diminished E-cadherin label indicated that the migratory potential of the cells may be enhanced by a reduction in cell–cell adhesion.

Each case was labelled on approximately serial sections to allow direct comparisons between each marker. Figure 1 shows an H&E-stained section of an excised lesion diagnosed as KA. Although Figure 1 bears a resemblance to a benign keratosis, an examination of the lesion itself and the other excised blocks resulted in a diagnosis of KA. The epidermal cells were strongly labelled for the cytolytic $P2X_7$ receptor (Figure 1b), indicating the initiation of a local apoptotic defence against abnormal cell proliferation. In normal skin these receptors are absent.[15] A high-power micrograph of the area demarcated by a box in Figure 1b shows the distribution of the $P2X_7$ label (Figure 1c,d). Depending on the plane of section of each cell, the cytolasmic and/or plasma membrane label may be seen as a ring (transverse section), partially or completely covering the cell (Figure 1d). TUNEL labelling in an approximately serial section (Figure 2a,b) shows that apoptosis in the same area shown in Figure 1a,b has proceeded to completion and that the cells are being destroyed. The area shown in Figure 2b is delineated by a box in Figure 2a. In the same area, anti-TP1 (Figure 3a,b) has a similar labelling pattern and intensity to that of the $P2X_7$ marker (Figure 1b). TP1 appears to be a cytoplasmic label (Figure 3b), as opposed to the plasma membrane label of $P2X_7$ (Figure 1d). The E-cadherin label in KA was strong and located on the plasma membrane of each cell (Figure 4a,b). This is an indication that the cells remain strongly attached to one another.

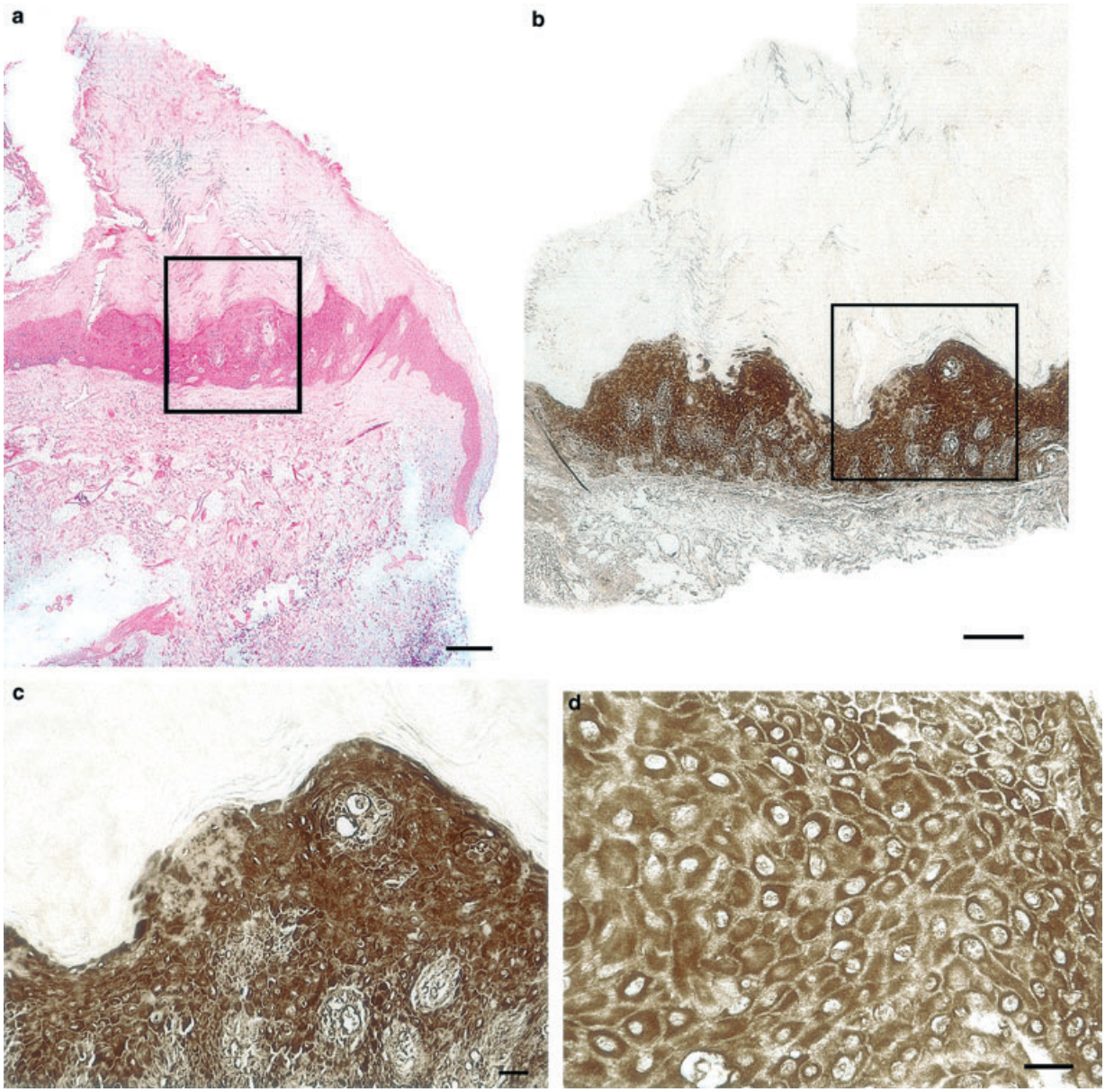

Figure 1. a, An H&E-stained section of an excised lesion previously diagnosed as a clear case of keratoacanthoma (KA). Note the eosinophillic nature of the stain, which is a feature of both KA and squamous cell carcinoma H&E-stained tissue. Bar = 0.25 mm. **b,** The epidermal cells in KA are strongly labelled for the cytolytic $P2X_7$ receptor, indicating the initiation of a local apoptotic defence. In normal skin these receptors are absent. Bar = 300 μm. **c,d,** A high-power micrograph of the area demarcated by a box in (**b**) shows the distribution of the $P2X_7$ label. **c,** Bar = 50 μm; **d,** bar = 20 μm.

In SCC (Figure 5a, H&E stain), no apparent TUNEL label was seen (Figure 5b), indicating that apoptosis had not proceeded to cell death. The initiation of a cytolytic response to neoplasia was apparent from a strong coextensive anti-$P2X_7$ label (Figure 6a,b) and an anti-TP1 label (Figure 7a,b), however. The location of this label was not only at the surface of the epidermis, but positive cell clusters were also found near the dermis (Figures 6a and 7a). The E-cadherin label in SCC (Figure 8a) was 3.1-fold ($P < 0.0001$) less intense than that found in KA.

In cases where it is difficult to differentiate between KA and SCC, the location of $P2X_7$ labelling alone may be useful in making a definitive diagnosis. Figure 9a shows such a case. The anti-$P2X_7$ (Figure 9b, arrows) and anti-TP1 label was present both superficially and

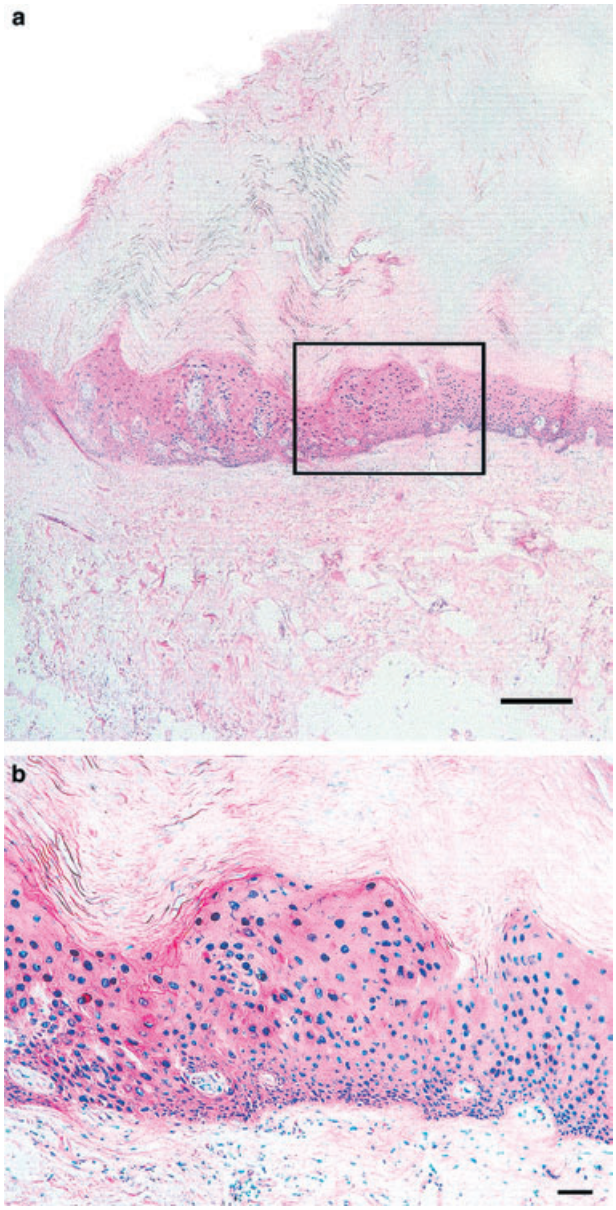

Figure 2. **a**, TUNEL labelling in an approximately serial section shows that apoptosis in the same area shown in Figure 1a–c has proceeded to completion and that the cells have been destroyed. Bar = 300 μm. **b,** This area is delineated by the box in (**a**). Bar = 50 μm.

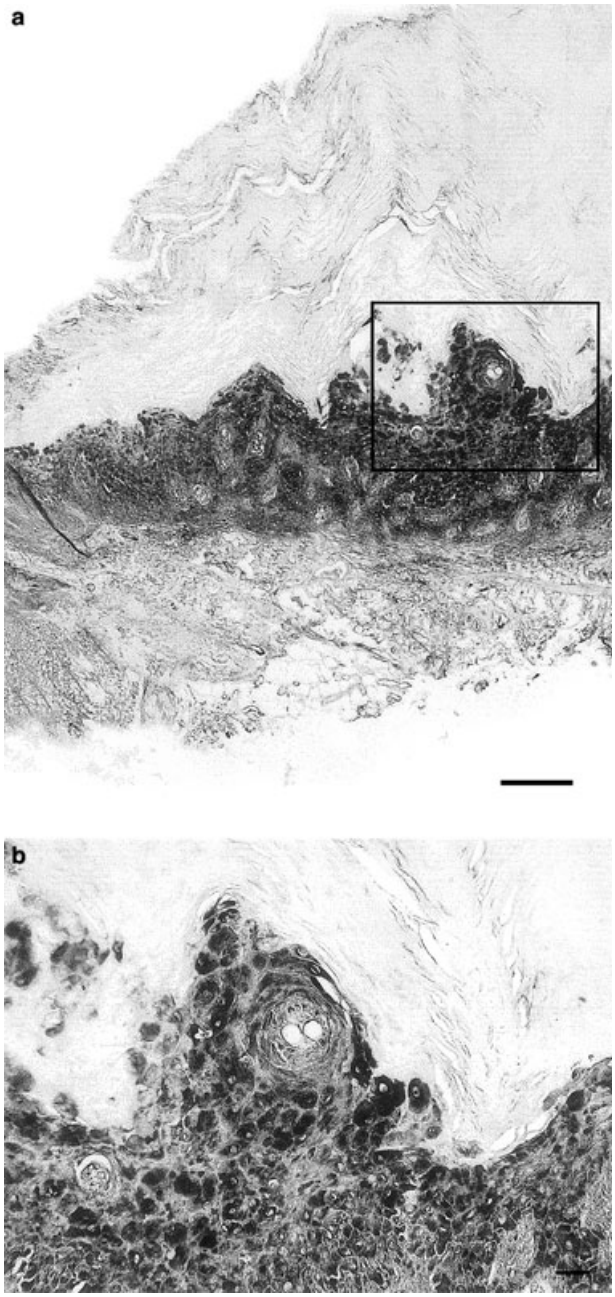

Figure 3. **a,b,** In the same area, anti-telomerase-associated protein has a similar intensity and labelling pattern to that of the $P2X_7$ marker in keratoacanthoma (Figure 1b–d). **a,** Bar = 300 μm; **b,** bar = 50 μm.

near the dermis. The E-cadherin label was patchy and weak and no TUNEL label was visible. Taken together, the labelling patterns of these markers indicated that this case was more typical of SCC than of KA. This case, and the other previously mentioned four cases that were difficult to diagnose, each had labelling features for anti-$P2X_7$, TUNEL, TP1 and E-cadherin that suggested they were in fact SCCs. Ultimately each of these lesions clinically progressed and required treatment, suggesting that this diagnosis with its prognostic implications was correct.

Discussion

In the current study, markers representing the initiation ($P2X_7$) and the end stage (cell death) of

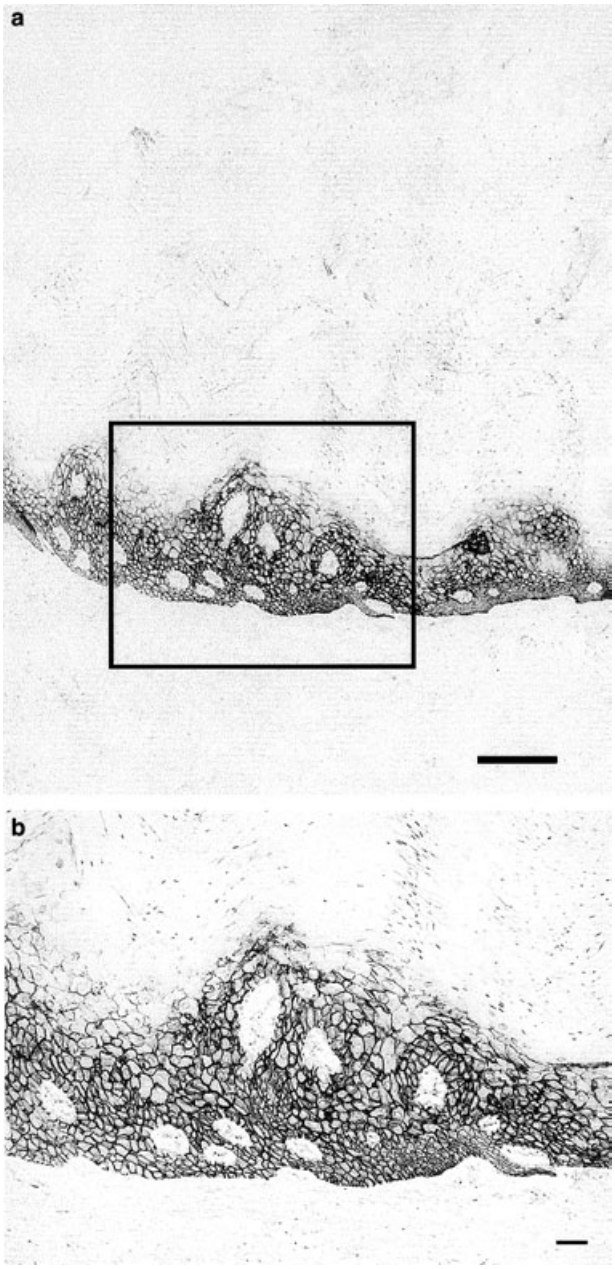

Figure 4. a,b, The E-cadherin label in keratoacanthoma is strong and located on the plasma membrane of each cell. This is an indication that the cells are strongly bound to one another. **a,** Bar = 300 μm; **b,** bar = 50 μm.

apoptosis, as demonstrated by TUNEL labelling, were both present in tissue diagnosed as KA. The proliferation enzyme TP1 was also present in these cells in the same areas and same intensity as the $P2X_7$ label. The cell adhesion protein E-cadherin labelled strongly in the plasma membrane of each cell, indicating strong cell adhesion.

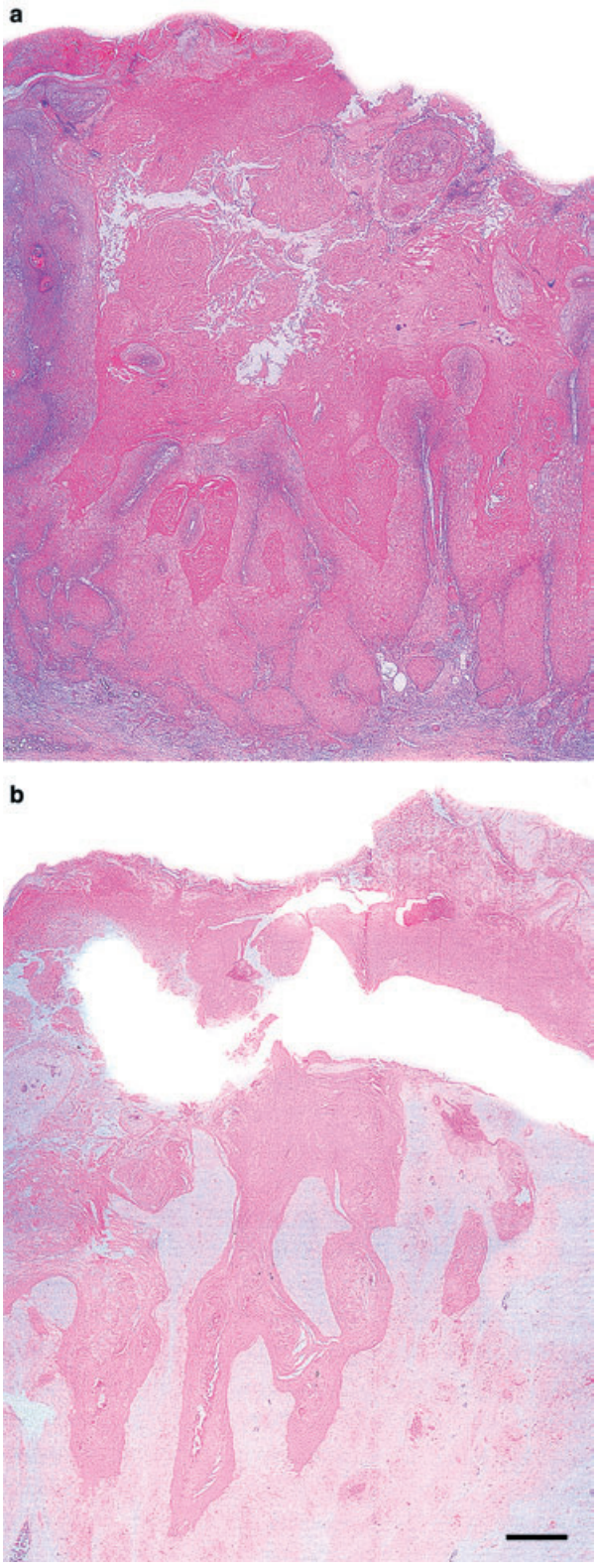

Figure 5. a,b, In squamous cell carcinoma (**a,** H&E stain), no apparent TUNEL label is seen (**b**), indicating that apoptosis has not proceeded to cell death. **b,** Bar = 500 μm.

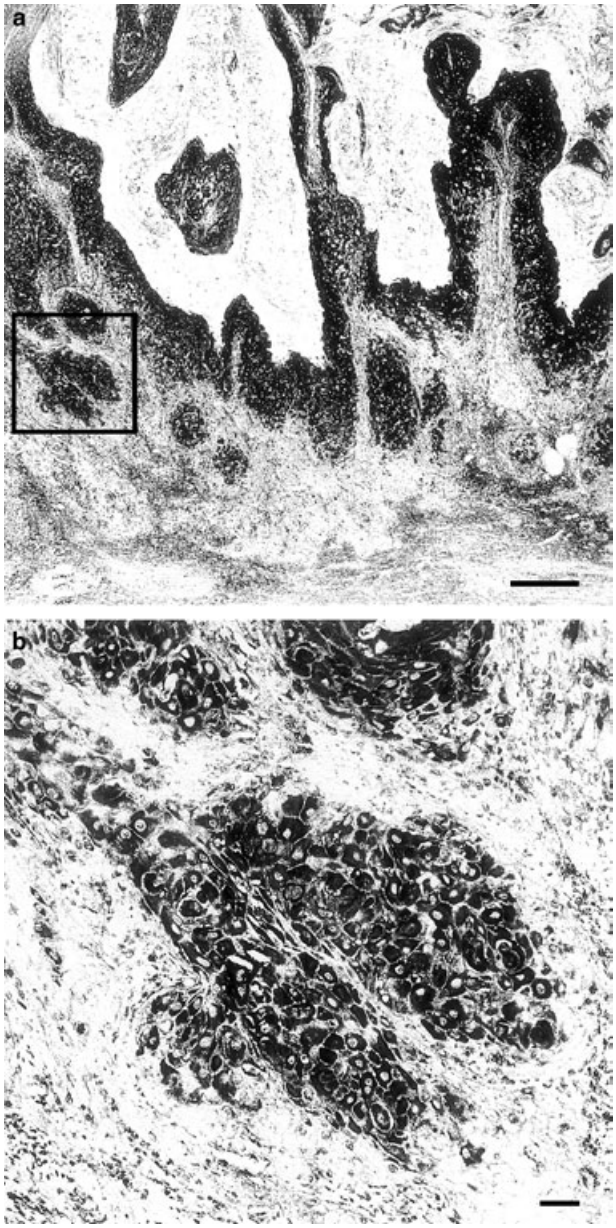

Figure 6. a,b, The initiation of a cytolytic response to neoplasia is apparent from the strong anti-$P2X_7$ label. The location of this label was not only superficial, as was the case in keratoacanthoma, but extended to near the dermis. **a,** Bar = 500 μm; **b,** bar = 50 μm.

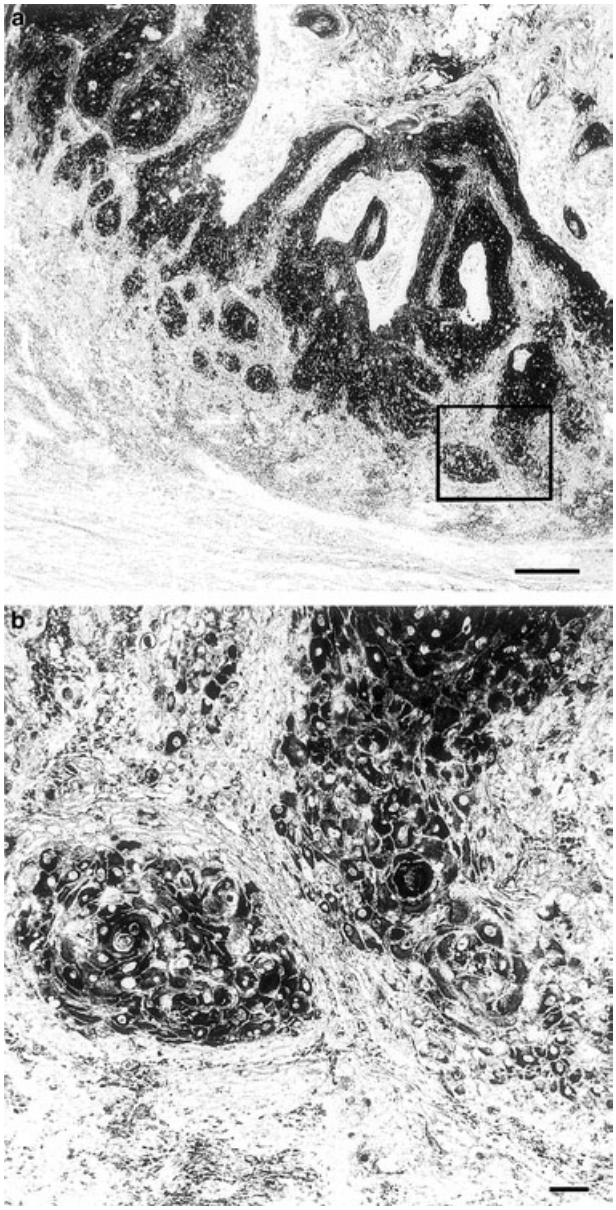

Figure 7. a,b, Anti-TP1 labelling is coextensive with the $P2X_7$ label; (**b**) shows a high-power image of the area delineated by the box in (**a**). **a,** Bar = 500 μm; **b,** bar = 50 μm.

In cases diagnosed as SCC, however, the initiation of apoptosis was also evident in the strong $P2X_7$ label, but the location of this label was quite different from that of KA. Instead of being confined to the surface epidermis adjacent to the keratin layer, both anti-TP1 and $P2X_7$ labelling in SCC commenced at the surface of the epidermis and continued to the dermis. The lack of TUNEL label in this tissue indicates that the apoptotic process was initiated but did not continue to completion. The E-cadherin label in SCC was significantly reduced, indicating a weakening of the bonds between cells and therefore an increased potential for invasion and possibly metastasis.

E-cadherin and catenins are adhesion molecules that are expressed identically in both normal skin and KA. Failure of E-cadherin and its associated proteins α-, β- and γ-catenin is believed to lead to disruption of cell–cell adhesion and to contribute to neoplasms such as SCC.[18] Our study supports this view, in that E-cadherin

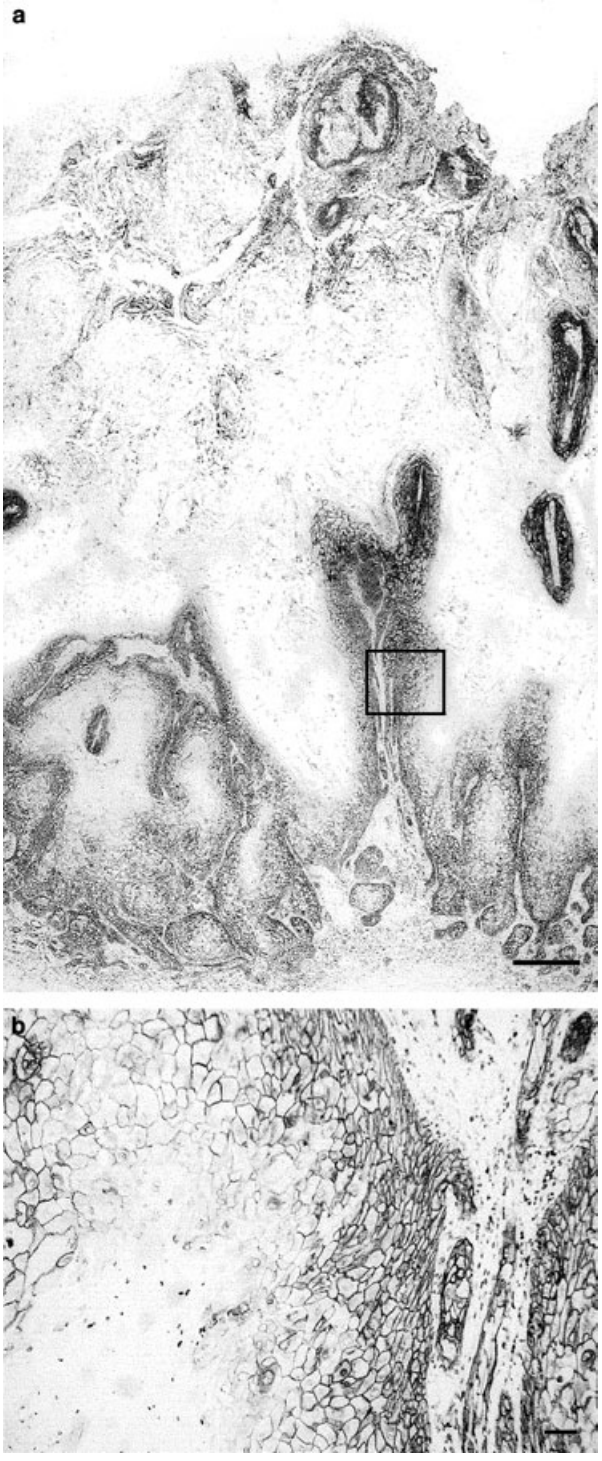

Figure 8. a,b, The E-cadherin label in squamous cell carcinoma was 3.1-fold less intense than that found in keratoacanthoma. **a,** Bar = 500 μm; **b,** bar = 50 μm.

labelling was strong in KA but was reduced 3.1-fold between SCC cells. The de-expression of cell adhesion molecules has previously been found to indicate

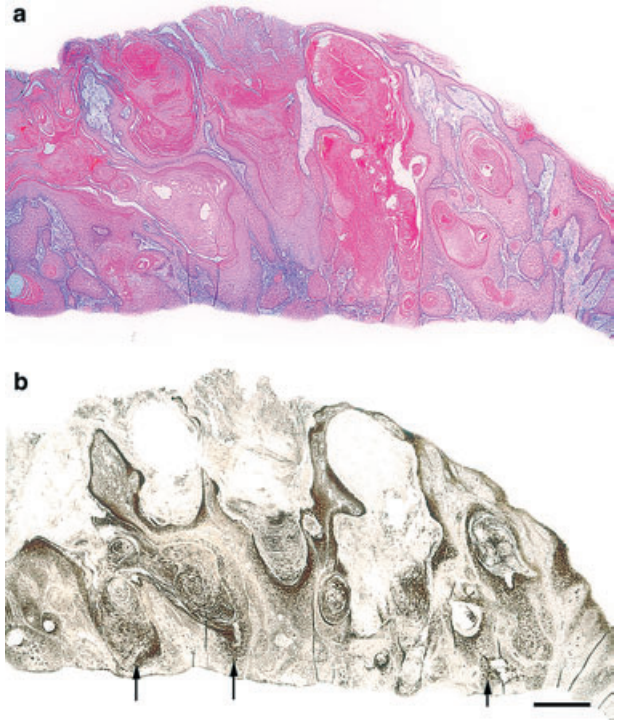

Figure 9. a,b, Using the distribution of anti-$P2X_7$ alone, the case shown is probably a squamous cell carcinoma rather than a keratoacanthoma (**b,** arrow). **b,** Bar = 1 mm.

tumour aggressiveness and invasiveness. Previous studies have shown that decreased levels of other cell adhesion molecules, such as syndecan-1, correlate with tumour de-differentiation.[9,19]

One school of thought suggests that KA is a separate entity from SCC. Proponents of this theory suggest that KA represents a proliferation of the infundibular portion of the hair follicles rather than the epidermis,[4] whereas SCC represents a malignant tumour of the epidermis. In the normal hair follicle, the cells have a programmed ability to be deleted by apoptosis. The current findings suggest that KA represents a temporary breakdown of the normal regulatory apoptotic mechanisms in the follicles. The TUNEL results suggest that these aberrant cells do undergo complete apoptosis, perhaps by another mechanism.

Taken together, these findings support the view that KA has a different pathogenesis and biochemistry from that of SCC, and is a different entity. The study also indicates that observing the labelling location of these markers, summarized by the cytolytic marker $P2X_7$, is an efficient way to differentiate KA from SCC where an initial differential diagnosis is difficult.

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