Increased expression of apoptotic markers in melanoma

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Extensive labelling for the apoptotic markers calcium channel receptor $P2X_7$ and caspase-3 and telomerase activity was co-localized at a similar intensity in areas affected by superficial spreading melanoma obtained from 80 patients. Labelling for each of these markers also extended 2μm from the melanoma into the keratinocyte layer of the adjacent normal epidermis. Conversely, the calcium-regulating receptors $P2X_{1-3}$ and $P2Y_2$ (found in normal but not neoplastic skin) were fully de-expressed within 2μm of the melanoma but fully expressed beyond that distance. The cell adhesion protein E-cadherin (also only present in normal skin) was progressively de-expressed from a point 2μm from the melanoma until full de-expression within the lesion. These results show that telomerase-induced proliferation and defensive apoptosis are co-localized and simultaneous processes in melanoma tissue. Melanoma cell proliferation appears to overwhelm the apoptotic defence, perhaps due to the anti-apoptotic effects of telomerase. In addition, keratinocyte regulation of the epidermis and dermis is severely compromised by the loss of E-cadherin and $P2X_{1-3}$ and $P2Y_2$ receptors, resulting in a lesion that is aggressive and malignant. *Melanoma Res* 13:137–145 © 2003 Lippincott Williams & Wilkins.

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Introduction

The incidence of malignant melanoma in many countries is increasing at a rate greater than that of any other cancer in humans [1]. Dysplastic naevi may be a precursor of melanoma in some cases. The early stages of melanoma are considered to be melanoma *in situ* and melanocytes in the radical growth phase (RGP). The advanced stages of the disease are primary melanoma in the vertical growth phase (VGP) and melanoma in the metastatic growth phase (MGP) [2].

The main defence against melanoma is programmed cell death (apoptosis), which is always involved in the spontaneous regression of neoplasms. Apoptosis also triggers the systemic immunological defences. Tumour treatments such as antineoplastic drugs, radiation and immunotherapy all include the initiation of apoptosis as part of their therapeutic action. Without apoptosis, tumour regression will not occur [3].

Most studies of melanoma have used melanotic cell lines, but the transformational biochemical changes that constitute early melanoma development are difficult to study in such models. Although VGP and MGP melanoma are available as cell lines, precursor lesions such as dysplastic naevi, melanoma *in situ* and RGP melanoma cannot be propagated *in vitro* [2].

In this study we used paraffin-embedded sections from 80 melanoma cases and 10 cases of normal skin to investigate apoptosis, P2X and P2Y receptor expression, telomerase activity, and E-cadherin cell adhesion protein changes in both normal skin and melanoma tissue. Serial sections were labelled using an amplified immunoperoxidase protocol. Expression of the apoptotic markers calcium channel receptor $P2X_7$ and caspase-3 or cysteine protease protein (CPP)-32, the ligand-gated purinergic calcium-receptor channels $P2X_{1-6}$, the metabotropic calcium receptor $P2Y_2$, the cell proliferation marker telomerase-associated protein, and the cell adhesion protein E-cadherin was studied in serial sections.

Materials and methods

A total of 80 cases of superficial spreading melanoma were examined. A small number of nodular and other histogenetic subtypes were also examined initially. Since the findings appeared to be similar in each of the various histogenetic subtypes tested, only cases of superficial spreading melanoma were chosen for the study, as this subtype best demonstrated both the depth and lateral extent of marker labelling.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections from previously diagnosed biopsies were used for this study.

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Each section was immunolabelled as previously described [4]. 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 in the appropriate primary antibody (anti-P2X, anti-P2Y or anti-human telomerase-associated protein [hTP1]). The concentration of affinity-purified IgG used in each case was 1.25mg/ml in phosphate buffered saline (PBS) for 30 min. The slides were washed three times in PBS for 5 min each, followed by a 30 min incubation with secondary antibody from the LSAB kit (Dako). All slides were then again washed in PBS for 5 min, visualized using a 0.05% solution of diaminobenzidine (DAB) for 5 min, washed, dried and mounted in Entellan mounting medium (Merck). Approximately serial sections were stained with a standard haematoxylin and eosin (H&E) protocol. Pre-incubation with peptide epitopes at 5–10 μM completely blocked labelling in the pre-absorption controls.

The sections labelled with monoclonal anti-E-cadherin (Zymed, San Francisco, California, USA) were treated with primary antibody at a concentration of 1:100 in PBS for 30 min. Human P2X and P2Y subtype-specific antibodies were used as described elsewhere [5–8]. The antibody titres, defined as the reciprocal of the serum dilution resulting in an absorbance of 1.0 above background in the enzyme-linked immunosorbent assay (ELISA), were all in the range 75 000–95 000 compared with 200–250 for the pre-immune samples.

Production of hTP1 antiserum

The consensus sequence of hTP1 [9] was examined for suitable epitopes. A segment in the C-terminal domain corresponding to the segment Cys2524–Glu2540 was chosen and the peptide synthesized using standard tertiary butoxycarbonyl (t-BOC) chemistry on an ABI 430A synthesizer (Applied Biosystems, Foster City, California, USA) [10]. After high performance liquid chromatography (HPLC) purification, the peptide was cross-linked to diphtheria toxin using maleimidocaproyl-*N*-hydroxysuccinimide. The peptide–antigen conjugate was suspended in water at a concentration of 5 mg/ml and aliquots were emulsified by mixing with Complete Freund's Adjuvant. Emulsion volumes of 1 ml containing 2 mg of peptide epitope were injected intramuscularly into a sheep; second and subsequent boosts were given at intervals of 6 weeks using Incomplete Freund's Adjuvant. Blood samples were obtained by venepuncture after 12 weeks when adequate antibody titres had been reached. The blood was incubated at 37°C for 30 min, and stored at 4°C for 15 h. The serum was then collected following centrifugation and stored at −20°C in small aliquots. The serum samples were tested with an ELISA assay. The titre of antibody used in the experiments was $82\,000 \pm 3300$ compared with 225 ± 25 for pre-immune serum. The antibody was affinity purified on Affi-Gel 10 (Biorad).

Results

Labelling for $P2X_7$, hTP1 and CPP32 was co-localized within the superficial spreading melanoma, while no labelling for these markers was detected in adjacent regions of normal skin in the melanoma samples or in the 10 separate sections of normal skin. In contrast, E-cadherin, $P2X_{1–3}$ and $P2Y_2$ were found in normal skin but not in melanoma.

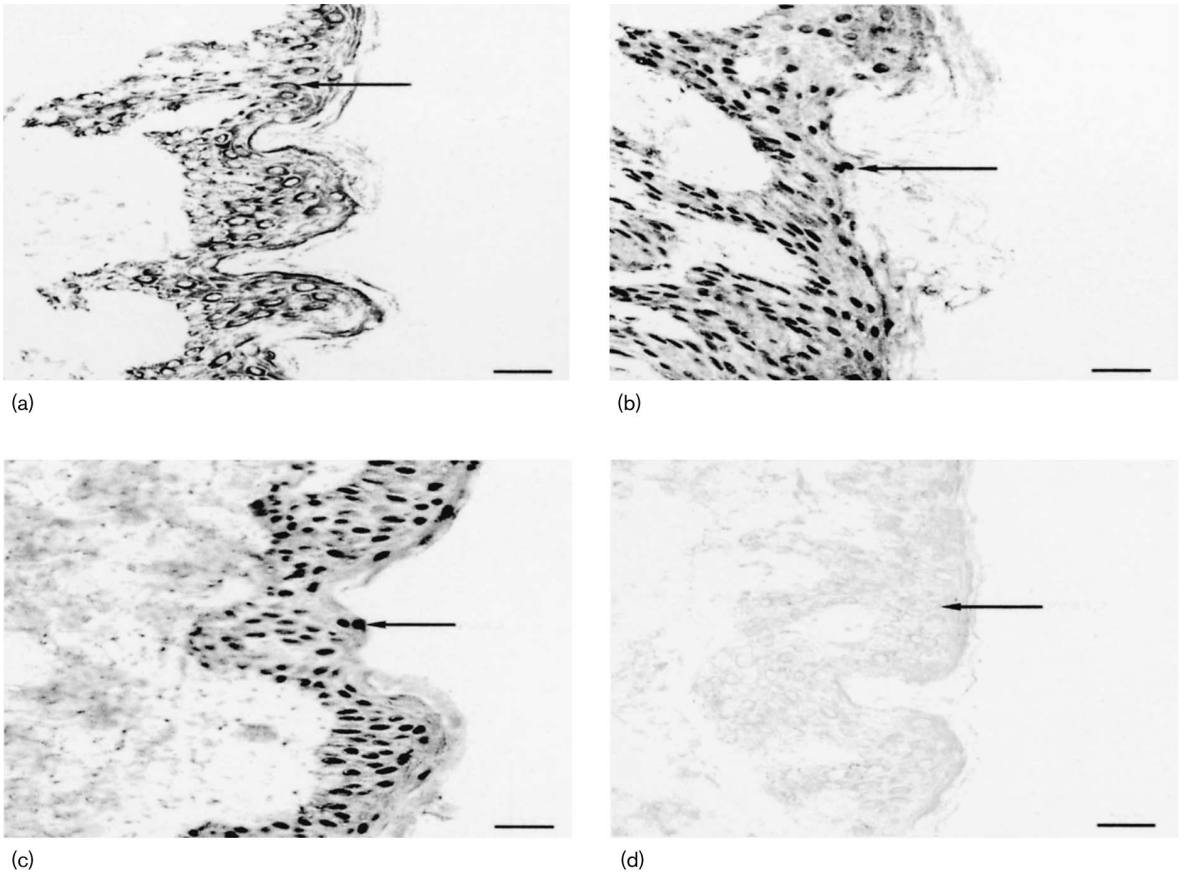
In the 10 normal skin cases and in the areas of normal skin not immediately adjacent to the melanomas, immunoperoxidase (IPX) labelling for the purinergic receptor $P2X_1$ took the form of distinct keratinocyte outlines (Fig. 1a). No label was observed in the stratum corneum. The bulk of the labelled cells were found in the stratum spinosum, with no particular concentration of labelled cells observed in the surrounding layers, either the stratum granulosum or the stratum basale. Strong nuclear labelling for $P2X_2$ (Fig. 1b) and $P2X_3$ (Fig. 1c) as well as the receptor $P2Y_2$ (results not shown) was also found throughout the stratum spinosum, with a sizeable proportion of all the epithelial cells in this layer exhibiting very similar intense labelling for all these receptors. $P2X_1$ labelling was relatively weak, with little apparent intracellular stain, indicating far lower protein expression than was evident for the other receptors. There was no detectable labelling for $P2X_{4–6}$ in either normal epithelium or in melanoma lesions (Fig. 1d). It was consistently evident that $P2X_2$, $P2X_3$ and $P2Y_2$ receptors were maximally expressed in normal skin further than 2 mm from the clear margins of a melanoma. An example of positive $P2Y_2$ labelling in normal epidermal cells is shown in Figure 2a. Within 2 mm of the clear margin of a melanotic lesion, however, all $P2X_{1–3}$ and $P2Y_2$ labelling was absent (Fig. 2b).

As the melanoma margins in these cases could be determined by H&E stain, it was possible to perform correlative microscopy and immunohistochemistry on approximately serial sections. Figure 3 shows H&E-stained sections of a well-defined melanoma (Fig. 3a) with normal epidermis on both borders (Fig. 3b). The arrow is a reference point 2 mm from any melanoma cells as defined by H&E labelling. Both sections show apparently normal epidermis at the edge of the melanoma.

IPX labelling for the cytolytic $P2X_7$ receptor (Fig. 4), the apoptotic marker CPP32 (Fig. 5a,b) and hTP1 (Fig. 5c,d) was co-localized and of similar intensity within the melanoma.

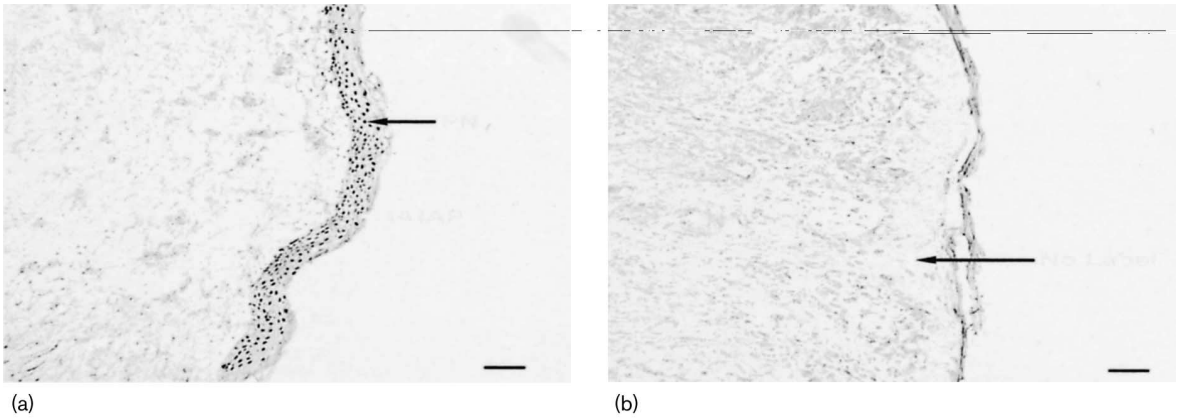
$P2X_7$, CPP32 and hTP1 were not labelled in normal

Fig. 1



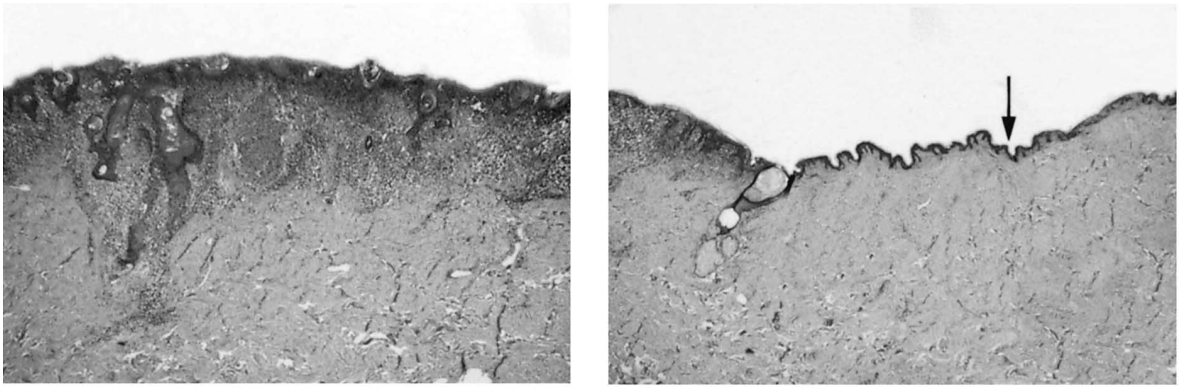
(a) IPX label for $P2X_1$ in normal epidermis. The label is in the form of nuclear outlines (arrow). (b) IPX label for $P2X_2$ in normal epidermis. An intense label occupies the nucleus of cells (arrow). (c) IPX label for $P2X_3$ in normal epidermis. The strong nuclear labelling pattern is very similar to that of $P2X_2$ (arrow). (d) IPX label for the P2X subtypes $P2X_{4-7}$ in normal epidermis. No label is present (arrow). Bar = 30 μm.

Fig. 2



(a) Low-power view of IPX labelling for $P2X_2$ in normal epidermis. Note that the label is positive and present all along the epidermis (arrow). (b) Within approximately 2 mm of melanoma cells, all labelling for $P2X_{2-3}$ disappears (arrow). Bar = 50 μm.

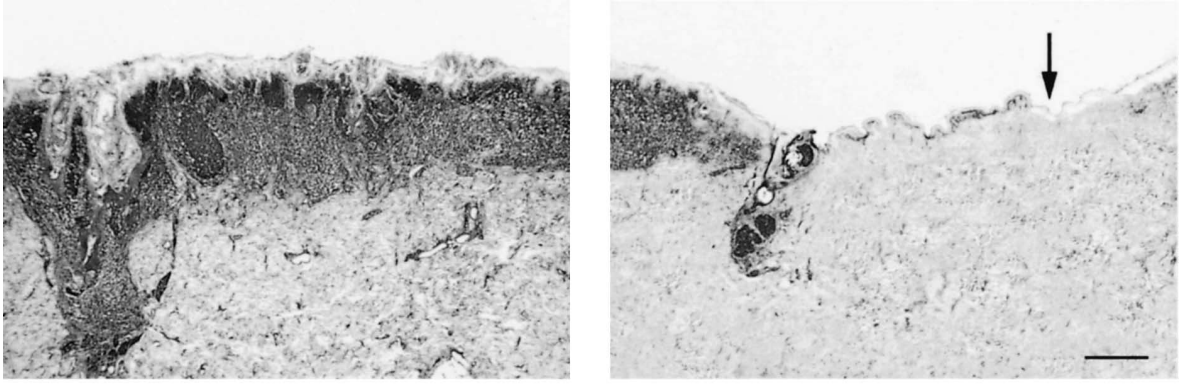
Fig. 3



(a) (b)

(a) H&E-stained section of an area of superficial spreading melanoma from the skin of the central back. (b) H&E-stained section of an area immediately adjacent to that shown in (a). About 75% of this micrograph shows normal skin. The point indicated by the arrow denotes the start of biochemically normal epidermis. No change is visible on H&E stain.

Fig. 4



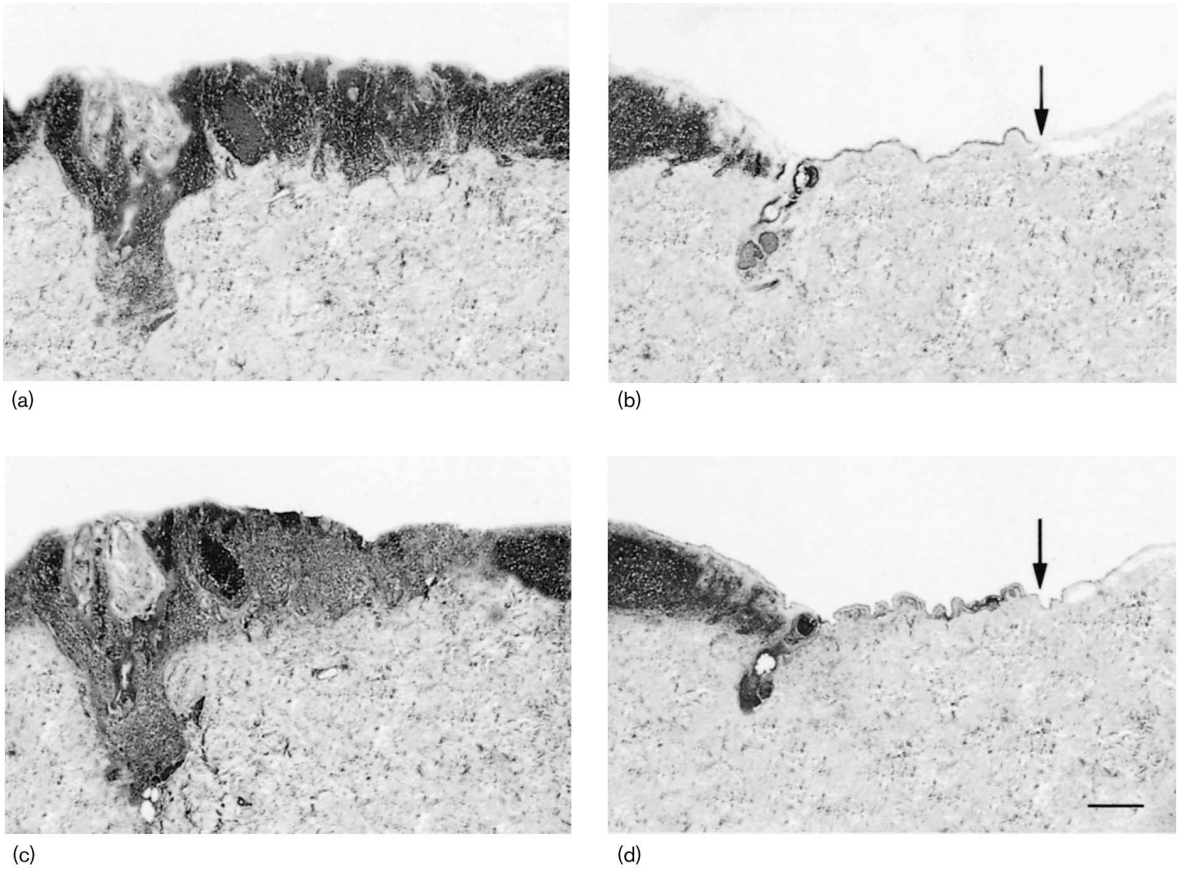
(a) (b)

(a) Approximately serial section showing IPX labelling for $P2X_7$. Expression of this cytolytic receptor/channel indicates that apoptosis is greatly upregulated in the melanoma. (b) Approximately serial section showing IPX labelling for $P2X_7$ in the area immediately adjacent to that shown in (a). Despite the apparently abrupt change between melanoma and normal skin as indicated by H&E stain (Fig. 3), some $P2X_7$ labelling extends past 2 mm from the melanoma into the adjacent, normal keratinocyte layer. From this point on (arrow), the skin does not show detectable $P2X_7$ expression and is probably biochemically normal. Bar = 0.5 μm.

skin in detectable amounts. In the melanoma tissue, as the labelling for $P2X_{1–3}$ disappeared within 2 mm of the tumour margins, the labelling for $P2X_7$, CPP32 and hTP1 progressively increased. Figure 4a shows labelling for $P2X_7$, indicating extensive expression of this receptor throughout the melanoma and extending out into the keratinocyte layer to a distance of approximately 2 mm (Fig. 4b). Figure 5a shows that the labelling of the apoptotic marker CPP32 correlates well with that of the $P2X_7$ receptors. There is an extensive and intense CPP32 label throughout the melanoma tissue that extends to the same reference point at 2 mm (Fig. 5b). Similarly, hTP1 is expressed with a similar intensity and distribution (Fig. 5c,d) as both $P2X_7$ and CPP32.

The cell adhesion protein E-cadherin was only expressed around keratinocytes. There was no detectable label for E-cadherin around normal melanocytes or melanoma cells. E-cadherin in the keratinocyte layer was de-expressed gradually with increasing proximity to a melanoma. Figure 6a is an H&E-stained section showing a well-defined melanoma with apparently normal adjacent skin that has been labelled with three reference points. There was no label for E-cadherin within the epidermis overlying the melanoma (Fig.

Fig. 5



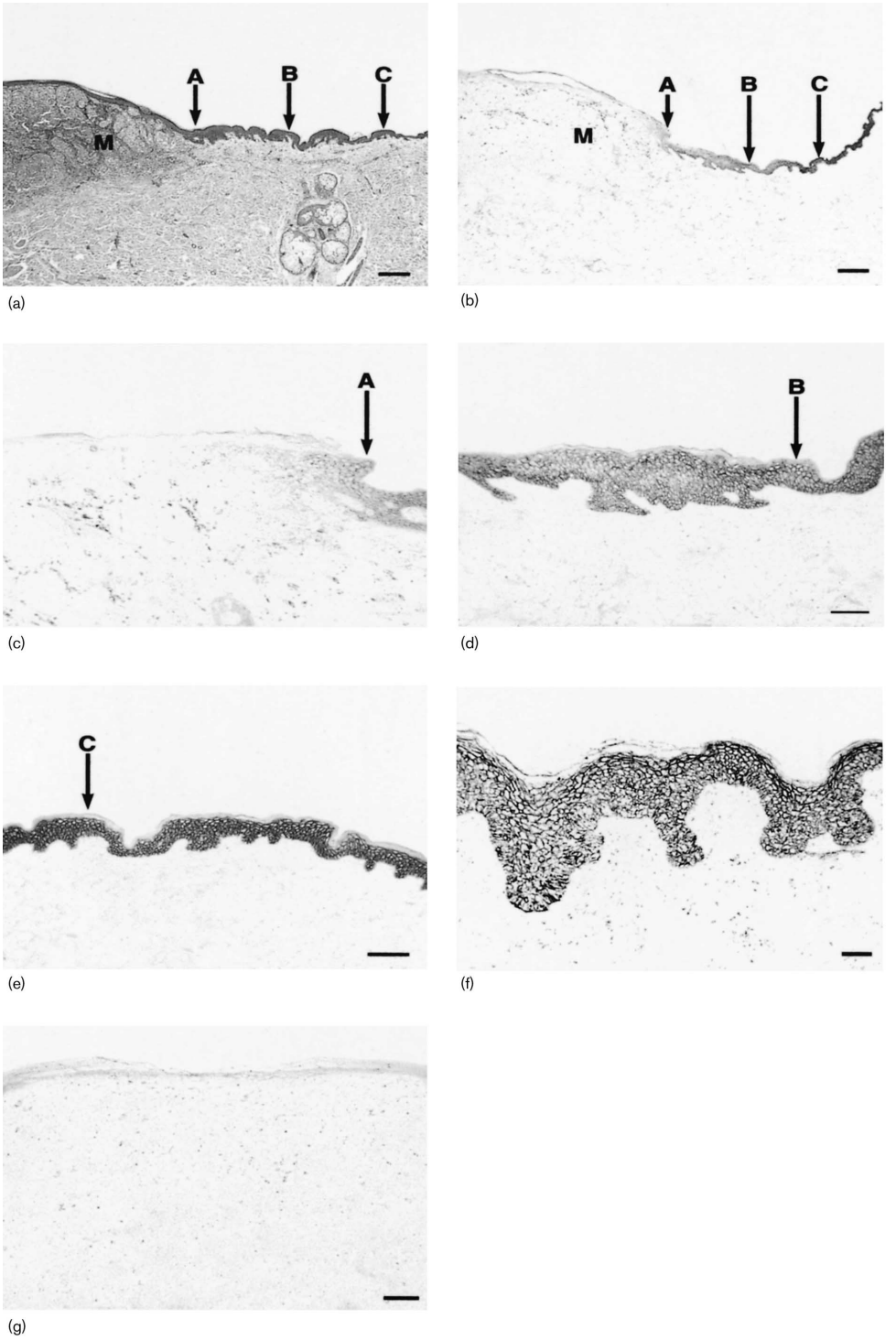
(a) Approximately serial section showing labelling for CPP32. Expression of this apoptotic marker is a further indication that apoptosis is widespread in the melanoma. (b) Approximately serial section showing IPX CPP32 labelling in the area immediately adjacent to that shown in (a). As with $P2X_7$ expression, some of the CPP32 labelling in the keratinocytes extends up to 2 mm from the melanoma cells. From this point on (arrow), the skin does not show detectable CPP32 expression and is probably biochemically normal. (c) Approximately serial section showing labelling for hTP1. As with $P2X_7$ and CPP32 labelling in this study, telomerase activity is widespread in the melanocytic areas. (d) Approximately serial section from adjacent to (c) showing IPX hTP1 labelling. As with $P2X_7$ and CPP32, there is extensive telomerase activity within the melanoma. This label extends in the keratinocyte layer up to 2 mm from the melanoma. Beyond this point (arrow), the skin is probably biochemically normal. Bar = 0.5 μm.

6a,b,c). However, immediately adjacent to the lesion some faint E-cadherin label was seen in the keratinocyte layer (Fig. 6c,d). The E-cadherin labelling increased to a moderate intensity approximately 1.2 mm from the lesion (Fig. 6b,d), but expression was not fully restored until a distance of $\geqslant 2$ mm of the melanoma (Fig. 6e). Figure 6f is a high-power micrograph of an area of maximal epidermal E-cadherin expression, while Figure 6g demonstrates full de-expression of E-cadherin in the epidermis directly adjacent to a melanoma.

Discussion

In the current study it was clear that there was extensive, co-localized and intense apoptotic and telomerase activity in melanoma, as revealed by the expression of CPP32, the cytolytic $P2X_7$ receptor and hTP1. Squamous epithelial cells (keratinocytes) are major sites for the biosynthesis of cytokines and growth factors. Keratinocytes are active cells with a 7 day turnover. They not only influence keratinization, but also the function of the underlying dermis. In turn, dermal cells release cytokines and proteases that regulate endothelial cells and keratinocytes. The coordinated function of multiple epidermal and dermal cell populations allows the skin immune system to respond rapidly and effectively to a wide variety of insults. Keratinocytes are the first line of defence in the skin immune system, and keratinocyte-derived cytokines are pivotal in mobilizing leukocytes from blood and for signalling other cutaneous cells. Cytokine-mediated cellular communication also enables dermal fibroblasts and endothelial cells lining the cutaneous vasculature to participate in immune and inflammatory responses [11]. In the current study, CPP32, $P2X_7$ and hTP1 were also expressed

Fig. 6



(a) H&E-stained section of another case in which normal skin is adjacent to a melanoma. Arrows A, B and C are reference points for parts (b) to (e). (b) IPX E-cadherin labelled detail of (a). This low power overview shows a complete de-expression of E-cadherin in most cells of the melanoma, with a faint (A), medium (B) and complete (C) progressive expression of E-cadherin occurring gradually until approximately 2 mm from the melanoma (C), from which point completely normal expression of E-cadherin in the keratinocytes of the epidermis is observed. Bar = 300 μm. (c) Medium-power micrograph of E-cadherin de-expression in the epidermis immediately adjacent to the melanoma. (d) Medium-power micrograph of partial E-cadherin expression at a distance of 1 mm from the melanoma (B). Bar = 100 μm. (e) Medium-power micrograph of normal E-cadherin expression from approximately 2 mm from the melanoma (C). Bar = 100 μm. (f) IPX E-cadherin labelled high-power micrograph of the labelling pattern of E-cadherin in the keratinocyte layer of the normal epidermis. Bar = 25 μm. (g) High-power micrograph of the labelling pattern of E-cadherin in the epidermis immediately adjacent to a melanoma. The label appears to be completely de-expressed. Bar = 25 μm.

in the keratinocyte layer. Each marker was expressed in the keratinocytes of apparently normal epithelium within 2 mm of a melanoma margin. Each was abruptly de-expressed at this distance from the melanoma margin, and none of these markers were expressed in the normal epithelium. This finding suggests that the biochemical changes associated with melanoma extend to a distance of 2 mm from the lesion.

Conversely, the cell adhesion protein E-cadherin was fully expressed on the plasma membrane of all keratinocytes in normal skin but was not expressed in normal melanocytes or melanoma cells to a detectable level. This finding suggests that melanocytes are more freely able to migrate than are keratinocytes, a factor that is important in malignancy. In the keratinocyte layer, E-cadherin was fully expressed beyond a distance of approximately 2 mm from the melanoma margin.

Cell–cell cross-talk mediated by cadherins and connexins results in coordinated regulation of cell growth, differentiation, apoptosis and migration. Abnormal expression of adhesion receptors and breakdown of intercellular communication appears to drive tumour development and progression [12]. In this study, loss of E-cadherin in the keratinocyte layer accompanied melanoma development. This frees melanocytic cells from the regulatory mechanisms of the keratinocyte layer, providing melanoma cells with an increased opportunity to migrate [13]. A key event in cancer metastasis is the transendothelial migration of tumour cells. Prior to migration, adhesion molecules are de-expressed at the endothelial junction, which is then penetrated by melanoma cell pseudopods. If the keratinocytes-dermal-endothelial cell regulation pathway is disrupted, as suggested by the current study, endothelial cells can assist the transmigration of tumour cells [14].

Like E-cadherin, the ligand-gated purinergic ionic calcium channels $P2X_{1–3}$ and the metabotropic calcium receptor $P2Y_2$ were expressed in the keratinocytes of normal skin but were abruptly de-expressed within a distance of approximately 2 mm from the melanoma. Intracellular calcium is known to be a potent regulator of apoptosis and androgen receptor expression [15]. The purinergic cytolytic calcium channel receptor $P2X_7$ is a major initiator of apoptosis in haemopoietic cells, as the activated receptor initiates the opening of a large pore in the plasma membrane. The resulting pore admits a large influx of ionic calcium, thus triggering apoptosis. The other P2X subtypes have differing characteristics. Each subtype modulates intracellular calcium in slightly different ways. The intracellular Ca^{2+} signal is transduced via more than 40 intracellular Ca^{2+}-binding proteins, including the S100 proteins, tenascin, calmodulin, integrin and annexin [16]. These calcium-bound proteins activate and regulate the cell cycle, protein secretion, composition of nuclear proteins, DNA transcription, apoptosis, adhesion protein binding, cell differentiation and phosphorylation [17]. Changes in cytosolic Ca^{2+} therefore control a wide range of cellular responses. Additionally, an increase in cytoplasmic calcium affects the binding of proteins to the cytoskeleton, induces the additional release of intracellular calcium [18], activates the Na^+/H^+ antiporter, increases cytoplasmic alkalinization [19], alters phosphatidylinositol metabolism [20] and activates protein kinases [21]. Calcium ions also trigger microtubule transport of membrane-bound organelles, exocytosis, and increases in nitric oxide synthase activity [22–26]. P2Y receptors increase intracellular Ca^{2+} levels by causing the release of Ca^{2+} from the non-mitochondrial, receptor-operated, intracellular calcium stores in the sarcoplasmic reticulum [27], whereas P2X receptors increase intracellular levels of Ca^{2+} by creating an ion-selective channel to the extracellular fluid [28]. Purines and pyrimidines can also either stimulate or inhibit proliferation depending on the extracellular microenvironment, the physiological state of the target cells, the cell cycle stage and the expression of P2 receptors [15].

Apoptosis can be initiated in a number of ways. Tumour necrosis factor (TNF) activates two distinct antiproliferative pathways including p38 mitogen-activated protein kinase (MAPK)-dependent cell cycle disruption and CPP32-mediated apoptosis [29]. Melanoma cell apoptosis is also regulated by endogenous nitric oxide resulting from inducible nitric oxide synthase activity [30]. The increase in intracellular calcium caused by proliferation of the $P2X_7$ receptors would not only initiate apoptosis, but also trigger increases in nitric oxide synthase activity [31], in turn inducing melanoma-specific apoptosis.

Telomerase labelling was a prominent feature of all the melanomas in the current study. Telomerase is a ribonucleoprotein enzyme that compensates for the progressive erosion of chromosomal ends, called telomeres. In most somatic cells telomerase expression is repressed and the telomeres progressively shorten after each cell division, causing cell senescence. Conversely, telomerase is active in most human cancers, maintaining the integrity of chromosome ends and representing an important step in cell proliferation, immortalization and carcinogenesis [32]. Telomerase plays a key role in carcinogenesis and is activated in most immortal cell lines and human cancers, including cutaneous melanoma [33]. Levels of telomerase activity and telomerase RNA in melanocytic lesions correlate well with clinical stage and could potentially assist in the diagnosis of borderline lesions [34]. The apparent lack of telomerase activity in a small percentage of reported cases is characteristic of the current antibodies that are based on human telomerase reverse transcriptase (hTERT)

epitopes. These appear to be easily masked or rendered conformationally unavailable. The antibody in the current study is based on a telomerase-associated protein, a more exposed epitope. In addition, a variable but significant percentage of apparently normal epidermis adjacent to a melanoma is positive for telomerase, indicating underlying biochemical cancer-associated changes that are not yet visible by H&E stain [32].

Links between telomerase activity, cell proliferation, apoptosis and expression of cell cycle regulators have not been extensively elucidated in cutaneous melanoma [33]. It has been proposed, however, that the regulatory mechanism controlling telomerase activity in melanoma relies on the transcription and alternative splicing of human telomerase reverse transcriptase [35]. In the current study, hTP1 and apoptosis markers were found in the same locations and with similar abundance. This probably indicates that apoptosis is very active as a defence against cancer cell proliferation, but that the neoplastic process overwhelms the apoptotic defence. There is also evidence that telomerase has an active anti-apoptotic role, as telomerase-expressing cells with elongated telomeres are resistant to apoptosis induced by hydroxyl radicals [36]. TNF also activates an anti-apoptotic mechanism in melanoma cells [29]. These findings may partly explain why apoptosis is ineffective as a defence against melanoma.

These results show that telomerase-induced proliferation and defensive apoptosis are co-localized and simultaneous processes in melanoma tissue. Melanoma cell proliferation appears to overwhelm the apoptotic defence, perhaps due to the anti-apoptotic effects of telomerase. In addition, keratinocyte regulation of the epidermis and dermis is severely compromised by the loss of E-cadherin and $P2X_{1-3}$ and $P2Y_2$ receptors, resulting in a lesion that is aggressive and malignant.

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