Melanocyte–keratinocyte interaction induces calcium signalling and melanin transfer to keratinocytes

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Summary

Physical contact between melanocytes and keratinocytes is a prerequisite for melanosome transfer to occur, but cellular signals induced during or after contact are not fully understood. Herein, it is shown that interactions between melanocyte and keratinocyte plasma membranes induced a transient intracellular calcium signal in keratinocytes that was required for pigment transfer. This intracellular calcium signal occurred due to release of calcium from intracellular stores. Pigment transfer observed in melanocyte–keratinocyte co-cultures was inhibited when intracellular calcium in keratinocytes was chelated. We propose that a 'ligand-receptor' type interaction exists between melanocytes and keratinocytes that triggers intracellular calcium signalling in keratinocytes and mediates melanin transfer.

Key words: melanocyte/keratinocyte/intracellular calcium/melanin transfer

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Introduction

Specialized epidermal cells – melanocytes, produce and package melanin within organelles – melanosomes and transfer them to neighbouring keratinocytes in the epidermis, resulting in visible skin pigmentation. The process of melanosome transfer is a unique biological process that is poorly understood. Various hypotheses such as release of melanosomes by melanocytes followed by their endocytosis into keratinocytes, direct inoculation (injection) of melanosomes into keratinocytes and keratinocyte–melanocyte membrane fusion have been proposed (Boissy, 2003; Nguyen and Wei, 2004). Methods exist in literature to study pigment transfer in vitro (Aspengren et al., 2006; Berens et al., 2005).

Certain cell surface molecules have been identified on keratinocytes that regulate the process of melanosome phagocytosis. Some of these include keratinocyte growth factor receptor and protease-activated receptor-2 (PAR-2) (Boissy, 2003; Cardinali et al., 2005). It has been previously shown that modulation of PAR-2 expression can influence the amount of melanosome phagocytosis, a process in which keratinocyte–melanocyte contact is essential (Seiberg, 2001; Seiberg et al., 2000). Some of the other processes believed to be involved in melanocyte–keratinocyte interaction are formation of dendritic processes and filopodia that extend from melanocytes towards keratinocytes and E-cadherin homodimer formation between melanocytes and keratinocytes (Scott, 2002; Scott et al., 2002; Tang et al., 1994). But the mechanisms involved in melanocyte–keratinocyte recognition are currently not known.

Cell–cell interaction is responsible for regulation of cell shape, cell motility and tissue structure. Protein complexes found at points of cell contact are integrated into cellular signalling pathways leading to cell adhesion, proliferation, differentiation and apoptosis (Braga and Harwood, 2001). These cellular processes are commonly mediated by intracellular calcium signal that is considered as the initial signalling event. It is known that calcium signalling controls growth, differentiation and apoptosis of epidermal keratinocytes (Gniadecki and Gajkowska, 2003). In this paper, we have studied the role of intracellular free calcium concentration ($[Ca^{2+}]_i$) in keratinocyte–melanocyte recognition and melanin transfer.

Results and discussion

To study the process of melanin transfer and the role of intracellular calcium in this phenomenon, an in vitro melanin transfer assay was developed. When B16 melanoma cells were cultured with A431 epidermoid keratinocytes, B16 cells attached to the keratinocyte monolayer within 2 h. Pigment transfer to keratinocytes was observed using Fontana–Masson stain after 24 h of co-culturing (Figure 1A). In these co-cultures, 60 ± 5% keratinocytes had pigment present. To eliminate the possibility that the apparent melanin transfer is a result

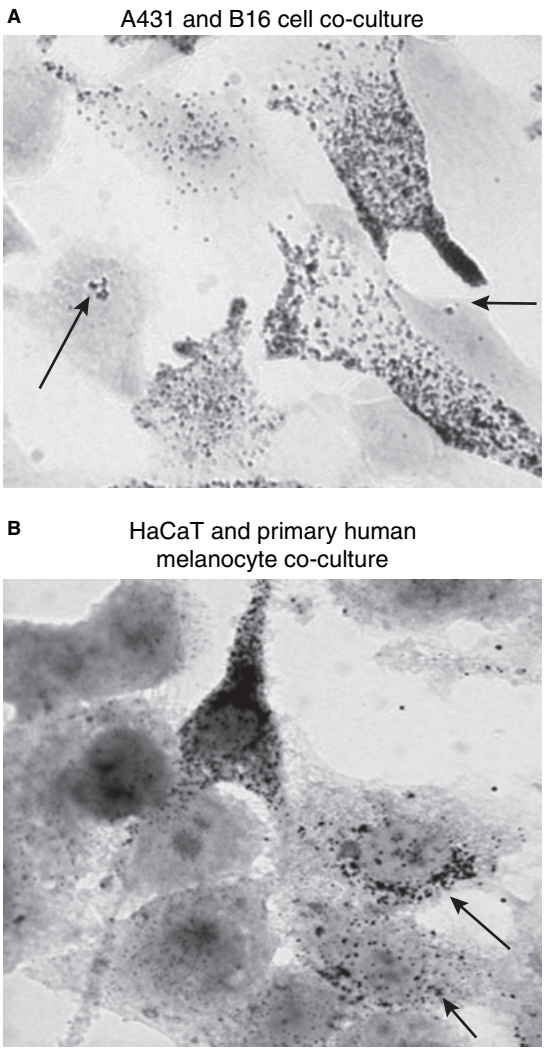

Figure 1. Pigment transfer in melanocyte–keratinocyte co-culture system. (A) A431 cells were co-cultured with B16 cells for 24 h. (B) HaCaT keratinocytes co-cultured with primary human melanocytes for 72 h. Cells were fixed and stained using Fontana–Masson stain. Arrows indicate pigment transfer.

of the transformed nature of the B16 cells or species heterogeneity, experiments were repeated using primary human melanocytes. In primary human melanocytes – HaCaT keratinocytes co-cultures, pigment transfer was observed, wherein 55 ± 5% keratinocytes had melanin present (Figure 1B).

The prerequisite for melanin transfer is contact between melanocytes and keratinocytes (Seiberg, 2001; Seiberg et al., 2000). We have attempted to understand melanocyte induced signalling events taking place in keratinocytes upon melanocyte–keratinocyte interaction. In melanocyte–keratinocyte co-cultures, melanocytes extend filopodia to keratinocytes, but technically it is not feasible to monitor intracellular signalling during the actual event of physical contact. Therefore, it was investigated whether isolated melanoma cell plasma membrane fraction induced signalling in keratinocytes. When plasma membrane isolated from B16 melanoma cells was added to A431 epidermoid keratinocytes, a transient increase in $[Ca^{2+}]_i$ was observed. Figure 2A,B show data from a typical experiment, wherein the $[Ca^{2+}]_i$ was monitored in n = 37 cells in a field. Upon addition of B16 cell plasma membrane, all the cells elicited a rise in intracellular calcium though the amplitude varied in different cells. The peak fluorescence intensity ratio varied from 1.0 to 1.6 in 200 cells from seven independent experiments on different batches of A431 cell cultures. The calcium ion (Ca^{2+}) signal induced by interaction of B16 plasma membrane appeared to be specific as no change in $[Ca^{2+}]_i$ was observed when A431 cells were exposed to plasma membrane isolated from other cell types, i.e. U87MG glioblastoma cells and primary cultures of rat brain astrocytes (Figure 2C,D). Melanocyte plasma membrane isolated without the presence of protease and protein tyrosine phosphatase inhibitors (phenylmethane sulfonyl fluoride and sodium orthovanadate) was unable to induce a Ca^{2+} response in A431 cells (Figure 2E). Similarly, when protein activity of the melanoma plasma membrane fraction was inhibited by repeated freeze-thawing, calcium signal was not observed in A431 cells (data not shown). These results imply that Ca^{2+} signalling in keratinocytes requires the presence of melanocyte membrane protein(s) in an active conformation. The rise in $[Ca^{2+}]_i$ in A431 cells occurred because of release of Ca^{2+} from intracellular stores, as a similar response was obtained even in the absence of Ca^{2+} in the extracellular buffer (Figure 2F). As seen in the A431-B16 co-culture system, addition of primary human melanocyte plasma membrane fraction to HaCaT keratinocytes also led to a transient increase in $[Ca^{2+}]_i$ in keratinocytes (Figure 2G). Further, the calcium signal induced by primary melanocyte plasma membrane fraction in HaCaT keratinocytes was also found to be due to release of Ca^{2+} from intracellular stores (Figure 2H). These experiments together show the reproducibility of $[Ca^{2+}]_i$ increase in the human system.

It was found that A431 cells once treated with B16 melanoma plasma membrane were unresponsive to second stimulation up to 2 h (Figure 3). This could possibly mean that there is a 'receptor-ligand-like' interaction, where desensitization of cell surface receptors is a common phenomenon. These data confirm that melanocyte–keratinocyte recognition is specific and suggest that such physical interaction leads to generation of an intracellular calcium signal in keratinocytes. Additionally, in the A431-B16 co-culture system, buffering intracellular calcium in A431 cells with calcium chelator–BAPTA-AM, significantly decreased pigment transfer (Figure 4) from B16 cells to A431 cells. These data suggest the role of melanocyte-induced calcium signal in keratinocytes in the process of melanosome transfer.

Cell–cell interactions are ubiquitous throughout biology and are critical for various events including tissue

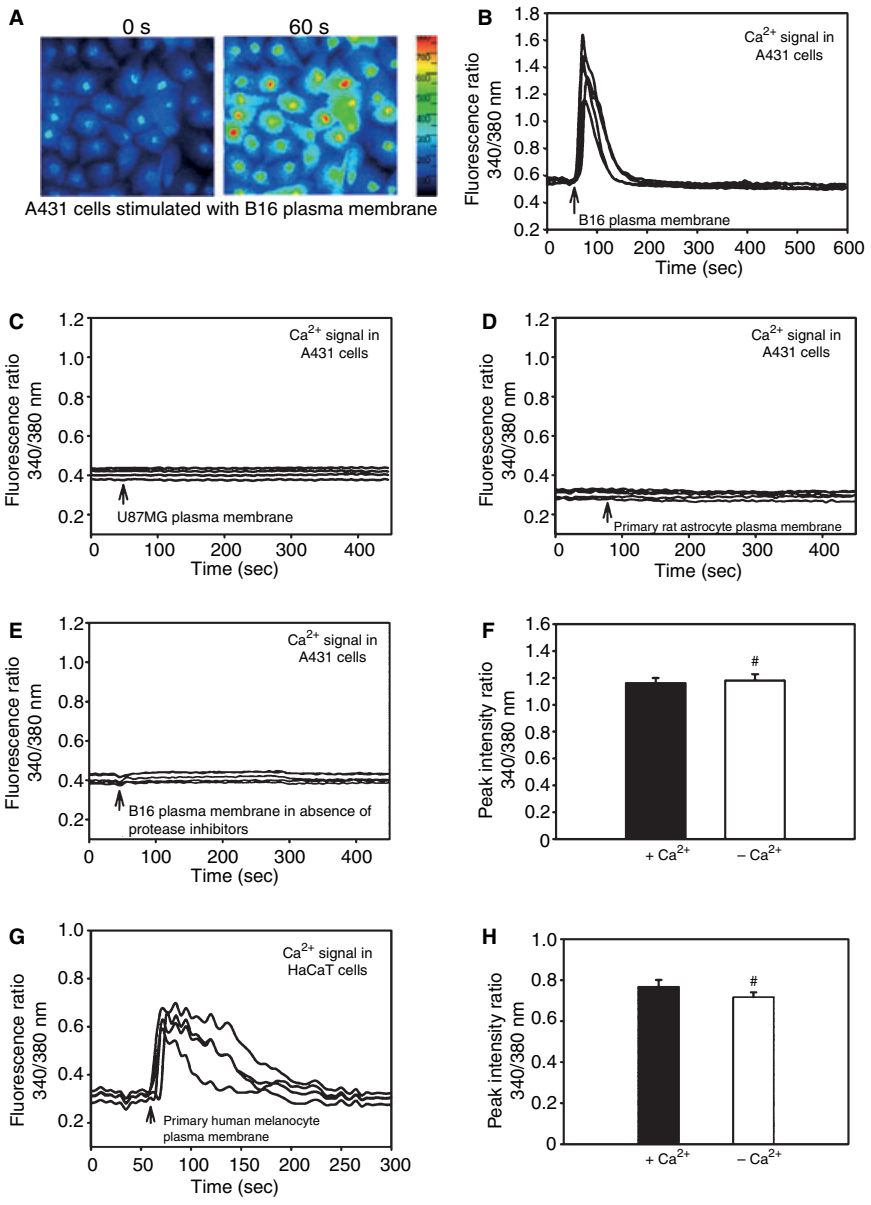

Figure 2. Melanocyte plasma membrane induced calcium signal in keratinocytes. (A) Pseudocolor ratio images (F340/F380) of A431 cells before (left panel) and 60 s after (right panel) addition of B16 plasma membrane (×1000). A431 cells (n = 30–40) were stimulated with plasma membranes isolated in presence of protease inhibitors from: (B) B16 cells; (C) U87MG cells; (D) primary rat astrocytes; and (E) B16 plasma membrane isolated in the absence of protease inhibitors. The data shown are representative traces of four cells in a single experiment repeated at least five times. (F) B16 membrane induced rise in $[Ca^{2+}]_i$ in A431 cells in the absence or presence (2 mM) of extracellular calcium. The data are mean ± SEM (n = 50) from three separate experiments. #P > 0.1. (G) HaCaT cells (n = 30–40) were stimulated with plasma membranes isolated from primary human melanocytes. The data shown are representative traces of four cells in a single experiment repeated at least three times. (H) Primary melanocyte membrane induced rise in calcium in HaCaT cells in the absence or presence (2 mM) of extracellular calcium. The data are mean ± SEM (n = 30) from three separate experiments. #P > 0.1.

construction and integrity, intercellular communication, information transfer, spatial awareness, normal cell growth and differentiation. Melanocyte–keratinocyte interaction is the basis of generation of skin colour. One of the most visible consequences of this interaction is the transfer of melanin from melanocytes to keratinocytes. In addition, keratinocytes affect other functions of melanocytes, including the process of melanin synthesis, melanocyte proliferation, etc (Duval et al., 2002; Nakazawa et al., 1995a,b; Valyi-Nagy et al., 1993).

Our data have shown that melanin transfer occurs in two different co-culture systems and involves a 'receptor-ligand-like' interaction that leads to intracellular calcium signals in keratinocytes. Intracellular calcium signalling induced by protein–protein interaction upon physical contact of two cell types has been described in other cell systems. For example, B cell activation by T cells (Kim et al., 2001) and control of axonal growth in neurons by myelin membrane (Hunt et al., 2002). NOGO or MAG protein(s) present on the myelin membrane bind to NOGO receptor on the neuronal growth cone membrane, and induce release of $[Ca^{2+}]_i$ and activate other signalling pathways, which in turn control axonal growth.

Bush and Simon (2007) have shown that melanosomes can sequester calcium and help maintain calcium homeostasis in melanocytes. In vivo, the possibility exists for calcium signals to be generated by keratinocytes through release of calcium from melanosomes transferred to keratinocytes. The data in this paper present strong evidence that interaction of melanocyte plasma membrane with keratinocytes induces calcium signalling within keratinocytes.

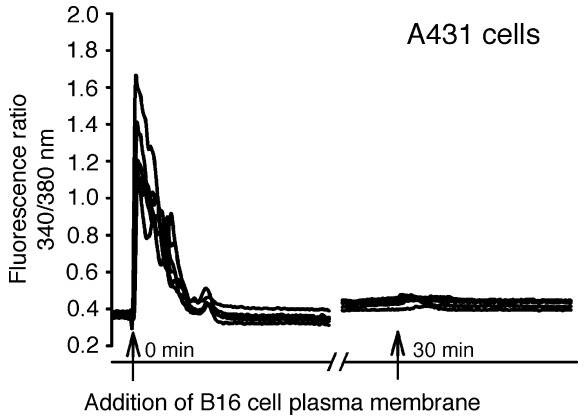

Figure 3. Desensitization of $[Ca^{2+}]_i$ signal in A431 cells. Cells were stimulated with B16 membrane as indicated by arrows. The second stimulation was after 30 min. The data shown are representative traces of four cells in a single experiment repeated at least three times.

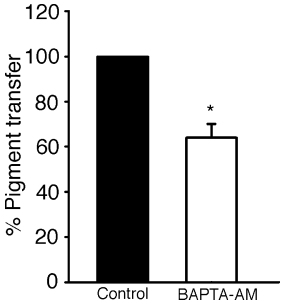

Figure 4. Decreased pigment transfer upon chelation of $[Ca^{2+}]_i$ in A431 cells. Data are shown as average ± SEM of five experiments (*P < 0.05).

Methods

Cell culture

HaCaT human keratinocytes were a kind gift from Dr Fusenig, Germany. U-87 MG human astrocytoma/glioblastoma cells, A431 human epidermoid cells and B16 mouse melanoma cells were obtained from the National Centre for Cell Science, Pune, India. HaCaT, U-87 MG, A431 and B16 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; procured from Sigma Chemical Co., St Louis, MO, USA) supplemented with 10 U/ml penicillin G, 0.1 mg/ml streptomycin sulphate, 25 mM HEPES buffer and 10% fetal calf serum (FCS; Gibco; Invitrogen Corporation, Grand Island, NY, USA).

Primary hippocampal astrocytes were obtained from 2 to 4 day-old Sprague Dawley rats as described by Koizumi et al., 2002. Briefly, cells were plated in Minimal Essential Medium with Earl's salts containing 10% fetal calf serum, 10 mM glucose, 50 U/ml Penicillin, 50 μg/ml streptomycin sulphate.

Neonatal foreskin primary human epidermal melanocytes (HEMn-DP) were procured from Cascade Biologics, Portland, OR, USA. These cells were grown in Medium 254 supplemented with human melanocyte growth supplement-2 (Cascade). All cell types were maintained at 37°C in a humidified incubator with 5% CO_2 atmosphere.

Fontana–Masson staining and melanin transfer assay

Cultured cells were washed thoroughly with phosphate buffered saline (PBS), pH 7.4 and fixed for 20 min with 4% formaldehyde. Cells were then stained using Fontana–Masson stain for 1 h at 55°C in the dark (Sheehan and Hrapchak, 1980). Cells were washed with distilled water and counter-stained with 0.1% eosin. Presence of pigment in keratinocytes was visualized by light microscopy using Olympus BX50 microscope. Images were captured using Image Pro-plus software with Cool-Snap CCD camera. Twenty-five random fields per plate were assessed and numbers of A431 cells containing melanin, in contact with B16 cells, were counted. Pigment transfer is represented as % control. Data are represented as average ± SEM of at least five experiments (*P < 0.05).

In the A431-B16 co-culture system, 4×10^4 A431 cells were first plated on 35 mm^2 Petri plates in DMEM with 10% FCS for 2 days. Following this, 2×10^4 B16 cells were added. Cells were fixed and stained using Fontana–Masson stain after 24 h. In the primary human melanocyte – HaCaT keratinocyte co-culture system, 2×10^4 primary melanocytes were first plated on 35 mm^2 Petri plates in primary melanocyte growth medium (Cascade). After 72 h, medium was replaced by serum free keratinocyte growth media (SF-KGM; Gibco, Grand Island, NY, USA) and 4×10^4 HaCaT keratinocytes were added to the plates. Cells were fixed and stained using Fontana–Masson stain after 72 h.

In some cases, A431 cells were treated with $[Ca^{2+}]_i$ chelator 1,2-bis-(o-Aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM; procured from Sigma Chemical Co.) on the third day. To load BAPTA-AM, DMEM media was replaced by SF-KGM. 25 μM BAPTA was mixed with 0.08% pluronic F-127 and added to A431 cells and cultures were incubated for 30 min at 37°C (Li et al., 1995). The cells were washed thoroughly with PBS and incubated in SF-KGM. B16 cells were then added and co-cultured for 24 h. Twenty-five random fields per plate were assessed and numbers of A431 cells containing melanin were counted. Pigment transfer is represented as % control. Data are represented as average ± SEM of at least five experiments (*P < 0.05).

Isolation of melanocyte plasma membranes

Plasma membrane was isolated from different cell types (as mentioned in the Results section). Cells were grown to confluency and harvested in cold PBS. Membranes were isolated as described previously (Sinha et al., 2003). The plasma membrane fraction obtained in the final pellet was resuspended in Tris sucrose buffer and protein concentration was estimated using Lowry method. The membrane preparations were aliquoted and stored at −70°C. Protease and protein tyrosine phosphatase inhibitors (100 μM sodium orthovanadate and 100 μg/ml phenylmethane sulfonyl fluoride) were used in the buffers throughout the preparation and storage, unless mentioned otherwise in the Results section.

Measurement of intracellular calcium

Change in $[Ca^{2+}]_i$ in cells was measured by real-time fluorescence imaging of cells as described previously (Lakshmi and Joshi, 2005; Sen et al., 2005). Cells were grown on poly L-lysine coated glass coverslips and washed with Ringer's buffer pH 7.2. Cells were loaded with 2 μM Fura-2 AM in Ringer's buffer containing 0.16% pluronic acid for 45 min at 37°C, washed three times with Ringer's buffer and kept in the dark for an additional 30 min to allow for complete deesterification of the dye. Ratiometric fluorescence imaging was performed with TILL Photonics imaging system (Germany) with Olympus Ix70 microscope using apo 40× objective. Cells were alternately excited at 340 and 380 nm and emitted fluorescence was

selected with appropriate filters. Recording was performed in calcium-free Ringer's buffer, unless otherwise mentioned in the results. Data acquisition and analysis was performed with TILL Vision software. Plasma membrane fractions, mentioned in the Results section, were added to the static bath (final protein concentration of ~25 μg, bath volume = 0.5 ml). Change in $[Ca^{2+}]_i$ is represented as change in ratio of fluorescence intensity at 340 and 380 nM. In each experiment, 20–30 cells in a field were imaged and each experiment was repeated three to fine times.

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