Role of melanoma chondroitin sulphate proteoglycan in patterning stem cells in human interfollicular epidermis

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Summary
Human interfollicular epidermis is renewed by stem cells that are clustered in the basal layer in a patterned, non-random distribution. Stem cells can be distinguished from other keratinocytes by high expression of β1 integrins and lack of expression of terminal differentiation markers; they divide infrequently in vivo but form actively growing colonies in culture. In a search for additional stem cell markers, we observed heterogeneous epidermal expression of melanoma chondroitin sulphate proteoglycan (MCSP). MCSP was expressed by those keratinocytes with the highest β1 integrin levels. In interfollicular epidermis, expression was confined to non-cycling cells and, in culture, to self-renewing clones. However, fluorescence-activated cell sorting on the basis of MCSP and β1 integrin expression gave no more enrichment for clonogenic keratinocytes than sorting for β1 integrins alone. To interfere with endogenous MCSP, we retrovirally infected keratinocytes with a chimera of the CD8 extracellular domain and the MCSP cytoplasmic domain. CD8/MCSP did not affect keratinocyte proliferation or differentiation but the cohesiveness of keratinocytes in isolated clones or reconstituted epidermal sheets was greatly reduced. CD8/MCSP caused stem cell progeny to scatter without differentiating. CD8/MCSP did not alter keratinocyte motility but disturbed cadherin-mediated cell-cell adhesion and the cortical actin cytoskeleton, effects that could be mimicked by inhibiting Rho. We conclude that MCSP is a novel marker for epidermal stem cells that contributes to their patterned distribution by promoting stem cell clustering.

Key words: Melanoma chondroitin sulphate proteoglycan, Epidermal stem cells, Patterning

Introduction
Mammalian epidermis is renewed throughout adult life by the proliferation of a subpopulation of keratinocytes known as stem cells. These cells are currently the subject of intense investigation and some general principles are beginning to emerge. First, it is widely agreed that, although stem cells have considerable (even unlimited) self-renewal ability, they divide infrequently in undamaged steady-state epidermis (Potten and Morris, 1988; Cotsarelis et al., 1990; Lavker and Sun, 2000). Nevertheless, when placed in culture they have the ability to find large, self-renewing clones consisting of a mixture of stem cells and terminally differentiating daughter cells (Barrandon and Green, 1987; Jones and Watt, 1993; Morris and Potten, 1994). Second, stem cells are multipotent and can generate daughter cells that differentiate into interfollicular epidermis (IFE), sebocytes and the multiple lineages of the hair follicles (Taylor et al., 2000; Fuchs et al., 2001; Oshima et al., 2001; Panteleyev et al., 2001; Niemann and Watt, 2002). Third, the distribution of stem cells is non-random. In hair-bearing skin, one reservoir of stem cells is a specialized region of the outer root sheath of the hair follicle known as the bulge (Taylor et al., 2000; Fuchs et al., 2001; Oshima et al., 2001). In human non-hair-bearing skin, cells with stem-cell characteristics (undifferentiated, infrequently cycling in vivo, clonogenic in culture) are clustered in a non-random distribution in the basal layer of the IFE (Jones et al., 1995; Jensen et al., 1999) (see also Asplund et al., 2001).

The clustering of stem cells in specific regions of the epidermis is interesting for two reasons. First, it suggests that there might be aspects of those regions (the stem cell microenvironment or niche) that are particularly conducive to the maintenance of the stem-cell phenotype (Watt and Hogan, 2000; Spradling et al., 2001). Second, it implies that movement of stem progeny within the epidermal basal layer occurs as part of the process of differentiation.

Cell migration has long been thought to play a role in maintaining the hair follicle through the hair growth cycle (Panteleyev et al., 2001), and lineage marking has unequivocally demonstrated the movement of cells from the bulge to the IFE and sebaceous glands (Oshima et al., 2001). Classically, movement of individual keratinocytes in IFE was thought to be restricted to the movement of cells upwards from the basal layer as they initiate terminal differentiation (Watt and Green, 1982), whereas lateral movement was restricted to
the migration of intact sheets of keratinocytes during wound healing (Martin, 1997). However, the clustering of stem cells in IFE implies lateral movement of stem cell daughters as they become committed progenitors (also known as transit amplifying cells). Evidence for the movement of individual keratinocytes relative to one another within the basal layer of IFE has come from analysis of green fluorescent protein (GFP)-marked clones in cultured sheets of human epidermis; here, GFP-positive committed progenitors are found intermingled with their unmarked neighbours, and GFP-labelled stem cells remain as cohesive groups (Jensen et al., 1999; Lowell et al., 2000).

The molecular basis of epidermal stem cell clustering is poorly understood. In both human and mouse epidermis, stem cells are more adhesive to extracellular matrix proteins than their nonstem progeny (Jones and Watt, 1993; Bickenbach and Chism, 1998) and, in human epidermis, this is due at least in part to high expression of β1 integrins (Jones and Watt, 1993; Jones et al., 1995; Jensen et al., 1999). There is also high β1-integrin expression in the bulge region of human hair follicles (Jones et al., 1995; Lyle et al., 1998; Akiyama et al., 2000). Elevated integrin expression results in reduced motility of both human keratinocytes (Jensen et al., 1999) and other cell types (Huttenlocher et al., 1995), and would therefore contribute to stem-cell clustering (Jensen et al., 1999). Human IFE stem cells also express higher levels of the Notch ligand Delta 1 than other basal cells, and this decreases keratinocyte motility via an unknown mechanism (Lowell et al., 2000; Lowell and Watt, 2001). One would predict that stem-cell clustering might also reflect increased intercellular adhesiveness, but although heterogeneity in expression of E-cadherin has been reported in vivo (Molés and Watt, 1997), this has not been confirmed in vitro (Zhu et al., 1999).

In a search for potential markers of human IFE stem cells, we noted that heterogeneous expression of melanoma chondroitin sulphate proteoglycan (MCSP) has been observed in the basal layer of human epidermis and in the outer root sheath of hair follicles (Kupsch et al., 1995). MCSP is so named because it is expressed in most human melanomas (Pluschke et al., 1996). MCSP is a cell surface proteoglycan that plays a role in the spreading, migration and invasion of melanoma cells (de Vries et al., 1986), stimulating α4β1-integrin-mediated cell adhesion and spreading via activation of the Rho family GTPase Cdc42 (Eisenmann et al., 1999). The rat homologue of MCSP is NG2 (Nishiyama et al., 1991); ligation of NG2 also results in cell attachment, migration and spreading, and reorganization of the actin cytoskeleton (Fang et al., 1999; Majumdar et al., 2003). Epidermal expression of MCSP is therefore of interest both as a putative stem cell marker and as a potential regulator of the adhesive properties, and thus spatial organization, of IFE keratinocytes.

Materials and methods

Construction of retroviral vectors and producer cell lines

The retroviral expression vector pBabepuro (pBp) (Morgenstern and Land, 1991) containing full-length CD8 (pBP-CD8) has been described previously (Bishop et al., 1998; Zhu et al., 1999). The MCSP cytoplasmic domain was obtained as IMAGE clone 40056 (I.M.A.G.E. Consortium) and amplified by PCR using the following oligonucleotide primers: 5'-AAGTCCTAGACGAAAAACCTGACGGG-3' (containing a XbaI restriction site, shown in bold) and 5'-GAGACCTCTAGAGCTGTC-3' (mutating base 7060 from A to C and creating a BamHI restriction site, shown in bold). The reaction conditions were ten cycles of 30 seconds at 95°C; 30 seconds at 58°C; 3 minutes at 68°C; 20 cycles of 30 seconds at 95°C; 30 seconds at 60°C and 3 minutes at 68°C, followed by 10 minutes at 72°C. The PCR fragment was digested with XbaI and BamHI, and cloned into the vector pCMUIV CD8β1 (Bishop et al., 1998), replacing the cytoplasmic domain of β1-integrin. The resultant construct (pBP-CD8/MCSP) encodes the extracellular and transmembrane domains of CD8β1, followed by a KYKSR linker derived from the first five amino acids of the cytoplasmic domain of the E3/19K adenosin protein, followed by the cytoplasmic domain (amino acids 2247-2322) of human MCSP (Pluschke et al., 1996) (accession number NM_001897).

Retroviral packaging lines were generated by a two-step procedure as follows. pBp, pBP-CD8 and pBP-CD8/MCSP DNAs were first transfected using a standard calcium phosphate transfection procedure (Sambrook et al., 1989) into the Phoenix ecotropic packaging line (Morgenstern and Land, 1991). Retroviral supernatant from the transfectants was used to infect the amphotropic retroviral producer line AM12 using a standard infection procedure (Morgenstern and Land, 1991). Infected cells were selected in the presence of 2 mg ml⁻¹ puromycin (Sigma).

For retroviral infection, keratinocytes were cultured in the presence of AM12 producer lines which had been pre-treated with 4 µg ml⁻¹ mitomycin C (Levy et al., 1998). The producer cells were removed after 3 days and replaced with puromycin-resistant J2-3T3 feeder cells, and 2 µg ml⁻¹ puromycin was added to the medium. Infected keratinocytes were used for experiments immediately or following passage on puromycin-resistant J2-3T3 cells in medium supplemented with 2 µg ml⁻¹ puromycin. Expression of constructs encoding CD8 in keratinocytes was determined by flow cytometry following labelling with monoclonal antibody (mAb) OKT8 (Zhu et al., 1999).

Keratinocyte culture

Normal human epidermal keratinocytes from neonatal foreskin (strain km; passages 2-8) were cultured as described previously (Watt, 1998) on a feeder layer of J2-3T3 feeder cells pre-treated with 4 µg ml⁻¹ mitomycin C (Sigma). The culture medium (FAD+FCS+HICE) consisted of one part Ham’s F12 medium and three parts Dulbecco’s modified Eagle’s medium, supplemented with 1.8×10⁻⁴ M adenine (FAD), 10% foetal calf serum (FCS), 0.5 µg ml⁻¹ hydrocortisone, 5 µg ml⁻¹ insulin, 10⁻¹⁰ M chola toxin and 10 ng ml⁻¹ epidermal growth factor (HICE). Cells were grown at 37°C in a humid atmosphere containing 5% CO₂. The culture medium was changed every 2 days. Cells were harvested by first removing the feeders with EDTA and then treating the keratinocytes with trypsin/EDTA. SCC4 cells, derived from a human squamous cell carcinoma of the tongue, were cultured as described previously (Evans et al., 2003). Preconfluent or newly confluent cultures were used for all experiments.

Antibodies and probes

MCSP was detected using mAb 9.2.27 (PharMingen), which recognizes both the nonglycated and proteoglycan forms of the molecule (Morgan et al., 1981; Bumol et al., 1984), or mAb LHM2 (Kupsch et al., 1995). For staining of whole mounts, mAb LHM2 was directly conjugated to Alexa-594. β1-Integrins were detected using either a FITC-conjugated anti-CD29 mAb (DAKO), mAb A1B2 (Bohnsack et al., 1990) or P5D2 (Dittel et al., 1993). E-Cadherin was detected using mAb HECED-1 (Shi moyama et al., 1989; Braga et al., 1995), CD8 was detected using mAb OKT8 (Reinherz et al., 1980a; Reinherz et al., 1980b) and involucrin using mAb SY5 (Hudson et al., 1992). The anti-Ki67 antigen Ab was a rabbit polyclonal antiserum.
collagen-coated dishes (BD Biosciences) at a density of 5
per dish for 5-8 hours at 37°C. Cells were removed using EDTA and
resuspended in HEPES-buffered DMEM (pH 7.5), 10% FCS and
0.5% BSA for 30 minutes at 4°C. Cells were then incubated sequentially with mAb 9.2,27, biotinylated anti-mouse Ig, RPE-Cy5-
conjugated streptavidin, mouse serum and FITC-conjugated CD29 mAb. Samples were washed three times between incubations and
finally resuspended in PBSABC containing 0.1% FCS. Fluorescence-
activated cell sorting (FACS) was performed using a MoFlo® High-
Performance Cell Sorter (DakoCytomation). Differentiating cells were gated out as described by Jones and Watt (Jones and Watt, 1993) to leave populations enriched for basal cells. Further gates were then applied to select populations expressing high and low levels of MCSP and β1 integrins. FACS-selected populations were analysed using a FACScan to check sorting efficiency and to quantify the expression levels of MCSP and β1 integrins in sorted populations.

**Clonogenicity assays**

Populations of uninfected keratinocytes selected on the basis of MCSP and β1 integrin expression were used directly from the FACS procedure. Retrovirus-infected keratinocyte populations were harvested using trypsin/EDTA. Keratinocytes were plated at 200, 500 and 1000 cells per dish, in triplicate, onto 6 cm diameter tissue-culture dishes containing mitotically inactivated J2-3T3 feeders and cultured for 10-14 days. Cells were fixed using 3% paraformaldehyde, permeabilized with 0.2% saponin and stained with 1% Rhodamine B and 1% Nile Blue (BDH). Colonies were viewed using a Wild 3BZ dissection microscope and scored as abortive or actively growing on the basis of size and proportion of terminally differentiated cells, as described by Jones and Watt (Jones and Watt, 1993).

**Function-blocking experiments with antibodies and pharmacological inhibitors**

Keratinocytes expressing CD8 or CD8/MCSP were incubated with the following pharmacological inhibitors for 6 hours at 37°C: 5 μM Y-27632 (Calbiochem) (Narumiya et al., 2000); 5 μg ml⁻¹ C3 exoenzyme from *Clostridium botulinum* (Calbiochem) (Udagawa and McIntyre, 1996); and 50 μM LY294002 (Sigma-Aldrich) (Pullen and Thomas, 1997).

Antibody crosslinking experiments were performed by incubating keratinocytes expressing either CD8 or CD8/MCSP with 50 μg ml⁻¹ of mAb OKT8 for 30 minutes, followed by 1 hour at 37°C with 50 μg ml⁻¹ of anti-mouse IgG. Control cultures were incubated with OKT8 alone, anti-mouse IgG alone or no antibody for 1 hour at 37°C.

To examine the effects on cell-cell adhesion of interfering with endogenous MCSP, intact sheets of SCC4 were cultured in low-calcium medium for 2 days to inhibit intercellular adhesion (Hodivala and Watt, 1994). Cells were then transferred to standard medium and incubated for 2 hours in medium alone, medium supplemented with anti-E-cadherin (80 μg ml⁻¹ HECD-1) and anti-MCSP (80 μg ml⁻¹ 9.2,27 or LHM2) antibodies (alone or in combination), or with rat NG2 ectodomain (30 μg ml⁻¹; generous gift of W. B. Stahlcup). Cells were fixed, permeabilized, stained with TRITC-phalloidin and viewed by confocal microscopy, as described above.

**Motility assays**

Retrovirus-infected keratinocyte populations were harvested with trypsin/EDTA and plated at a density of 1×10⁶ cells per dish onto type-I-collagen-coated 6 cm diameter tissue culture dishes (BD Biosciences) in FAD+FCS+HICE. Cell migration was recorded overnight at a 5 minute time lapse-interval using a 10x objective and the images were acquired using AQM Kinetic Acquisition Manager software (Kinetic Imaging) or by video microscopy. Analysis of motility was performed by manually tracking cells within each field over the sequence of time-lapse digital images using Motion Analysis software (Kinetic Imaging). All the individual cell trajectories were then pooled and mean speed of cells at each time point during the assay calculated. The average speed of cell motility was calculated for each assay and compared over four independent experiments. Results were compared using analysis of variance.
Time-lapse video microscopy of keratinocyte colonies

Retrovirus-infected keratinocyte populations were harvested with trypsin/EDTA, plated onto mitotically inactivated feeder cells in 6 cm dishes and cultured until small colonies appeared. Feeder cells were removed with EDTA and fresh culture medium added. Cells were allowed to recover for 30 minutes and then analysed by time-lapse video microscopy. Images were acquired at time lapse intervals of 5 minutes over a period of 68 hours.

Assays of keratinocyte cohesiveness

Populations of retrovirus-infected keratinocytes were harvested with trypsin/EDTA. 200 CD8- or CD8/MCSP-expressing keratinocytes were mixed with $1 \times 10^5$ keratinocytes infected with the empty retroviral vector, plated on mitotically inactivated feeder cells in six-well tissue-culture plates and cultured for 10-14 days to reconstitute confluent epidermal sheets. Clones of CD8 or CD8/MCSP-expressing keratinocytes within the sheets were detected by immunostaining with the anti-CD8 mAb OKT8. Fluorescence and phase-contrast images of these clones and surrounding unlabelled keratinocytes were acquired using a Nikon Diaphot 200 microscope attached to a Hamamatsu digital camera and AQM Kinetic Acquisition Manager software (Kinetic Imaging). Clones were scored as cohesive if they contained more than 70% of cells in contact with one another, whereas noncohesive clones contained fewer than 70% of cells in contact.

Results

Characterization of MCSP expression in human IFE

To determine the pattern of expression of MCSP in human skin, we used mAbs 9.2.27 (Morgan et al., 1981; Bumol et al., 1984) and LHM2 (Kupsch et al., 1995). In frozen sections of human non-palmoplantar skin from all body sites examined (scalp, breast, finger, foreskin), MCSP was expressed in the epidermis and was not detected in the dermis (Fig. 1A,B). In IFE, MCSP expression was confined to a subset of keratinocytes within the basal layer; these cells were found predominantly at the tips of the dermal papillae, where the basal layer of the epidermis is closest to the skin surface. In areas of the basal layer between the dermal papillae (the rete ridges), MCSP expression was markedly downregulated or undetectable (Fig. 1A).

Previous studies have demonstrated that clusters of basal keratinocytes situated at the tips of the dermal papillae of non-palmoplantar human epidermis express markers of epidermal stem cells, such as high levels of β1 integrins (Jones and Watt, 1993; Jones et al., 1995; Jensen et al., 1999). We therefore used double-label immunofluorescence to compare the expression of β1 integrins and MCSP. MCSP positive keratinocytes closely co-localized with areas of high β1 integrin expression and the proteoglycan was largely absent from basal keratinocytes with lower levels of β1 integrins (Fig. 1B).

To evaluate MCSP expression further, we used the technique of whole mount immunofluorescence labelling, in which the epidermis is detached as an intact sheet from the underlying dermis, fixed, incubated with the relevant antibodies and then viewed en face with a confocal microscope. Visualized by this technique, β1 integrin-bright keratinocytes appear as discrete clusters surrounded by more differentiated, integrin-dull cells (Fig. 1C) (Jensen et al., 1999).
labelling for MCSP showed that the clusters of MCSP-positive basal cells co-expressed high levels of β1 integrins (Fig. 1C). Actively cycling basal cells have previously been shown to lie in the integrin-dull basal compartment (Jensen et al., 1999). As predicted, MCSP-positive keratinocyte clusters tended to be negative for the proliferation marker Ki67 and, conversely, Ki67-positive basal cells were largely MCSP negative (Fig. 1D).

The expression of MCSP by clusters of β1-integrin-bright, nonproliferating keratinocytes suggested that MCSP might be a novel marker of stem cells in human IFE.

**Heterogeneous expression of MCSP by primary human keratinocytes in culture**

When primary human keratinocytes are cultured at low density on a feeder layer of mitotically inactivated 3T3 cells, different types of clone can be observed (Barrandon and Green, 1987; Jones and Watt, 1993; Jones et al., 1995). At 14 days after plating, large clones consisting of a mixture of dividing and terminally differentiated cells can be distinguished from small, abortive colonies in which all or most cells have initiated terminal differentiation (Jones and Watt, 1993). Stem cells found the former type of colony, whereas the abortive clones can be attributed to transit amplifying (committed progenitor) cells (reviewed by Watt, 1998). We therefore examined MCSP expression in clones of primary human IFE keratinocytes.

As in IFE, MCSP expression was heterogeneous in culture (Fig. 2A-C). Abortive, terminally differentiated colonies usually lacked any MCSP, even though the basal cells expressed β1 integrins (Fig. 2B). By contrast, proliferative keratinocyte colonies contained a mixture of MCSP-positive and -negative cells, the positive cells expressing higher levels of β1 integrins than the negative cells (Fig. 2A). In the youngest colonies (<7 days after plating), MCSP expression was largely concentrated in the peripheral basal cells (Fig. 2A), whereas in more mature clones MCSP-positive cells were scattered throughout the basal layer (Fig. 2C).

We also examined the subcellular distribution of MCSP. Antibodies to the proteoglycan strongly labelled cell-cell borders and the apical surface of keratinocytes, where it was present on the microvilli (Fig. 2C,E). By contrast, there was no colocalization of MCSP with focal adhesions (Fig. 2D).

To compare the expression of MCSP and β1 integrins quantitatively, we performed flow cytometry on disaggregated cultures of human keratinocytes (Fig. 3). The extracellular domain of MCSP was sensitive to the trypsinization procedure used to generate single cell suspensions (data not shown) (Nishiyama et al., 1995). Therefore, it was necessary to replate the cells on collagen-coated dishes for 5-8 hours to allow newly synthesized MCSP to reach the cell surface, and then to detach the cells with EDTA alone. The MCSP profile obtained from basal keratinocytes was more heterogeneous than the β1 integrin profile (Fig. 3B,C, ‘Total’). This correlates with the greater variation in MCSP levels between cells (Fig. 2A), although it might also reflect different rates of export of newly synthesized MCSP to the surface of cells following replating.

20% of basal cells with highest β1 integrin fluorescence (β1-High gate in Fig. 3A) (Jones and Watt, 1993). This fraction had a sevenfold higher mean MCSP expression than the 20% of keratinocytes with lowest β1 integrin expression (Fig. 3B, compare β1-High and β1-Low). The peak MCSP fluorescence of the low integrin expressing population was the same as the secondary antibody control (dotted line), indicating that the
cells were MCSP negative (Fig. 3B). Conversely, keratinocyte populations selected on the basis of high MCSP expression had a 1.8-fold enrichment in mean β1 integrin expression compared with populations selected for low MCSP expression (Fig. 3C, compare MCSP-High and MCSP-Low).

We conclude that the correlation between high MCSP expression and high β1 integrin expression observed in IFE (Fig. 1) extends to cultured IFE keratinocytes, and that it is possible to use FACS to select keratinocytes that are MCSP positive or negative.

Selection of high expressing keratinocytes gives no further enrichment for clonogenic cells

We used FACS to investigate whether selection of MCSP-positive, β1 integrin bright keratinocytes gave more enrichment for clonogenic human keratinocytes than selection on the basis of β1 integrin expression alone. Terminally differentiated cells were gated out as described previously (Jones and Watt, 1993) and basal keratinocytes were fractionated into the following four populations: the 20% of cells with the lowest β1-integrin expression (β1-Low); the cells in the highest 20% of β1-integrin expression and negative for MCSP (MCSP-Low/β1-High); cells within the highest 20% of β1-integrin expression and with high expression of MCSP (MCSP-High/β1-High); and, as a control, unselected basal keratinocytes (Total) (Fig. 4A).

Cells from each population were plated at clonal density onto mitotically inactivated fibroblast feeders and cultured for 10-14 days (Fig. 4B). No differences between the proportions of adherent cells were observed between the MCSP-Low/β1-High and MCSP-High/β1-High keratinocytes (data not shown). Clones of keratinocytes from each population were scored for colony forming efficiency (CFE; the proportion of plated cells that formed clones of any type) and the proportion of clones giving rise to abortive, terminally differentiated clones (Ab). Keratinocytes expressing low levels of β1 integrins gave rise to fewer colonies than the unselected population (CFE of 0.7% and 9.9% for β1-Low and Total, respectively) and to a higher proportion of abortive colonies (%Ab of 92% and 59% for β1-Low and Total, respectively). As reported previously (Jones and Watt, 1993), the high-β1 integrin population gave rise to fewer abortive colonies than the unselected population; however, this was true regardless of whether the cells expressed MCSP (%Ab of 48% and 43% for MCSP-Low/β1-High and MCSP-High/β1-High, respectively, compared with a %Ab of 59% for Total).

**Fig. 3.** Comparison of surface levels of MCSP and β1 integrins in primary human keratinocytes by flow cytometry. Profiles of basal keratinocytes are shown following gating out of differentiated cells. (A) Dot plot of total basal keratinocyte population. Vertical and horizontal lines represent the fluorescence of single antibody controls. Boxes represent cells selected on the basis of highest or lowest 20% of expression of β1 integrins (FL-1) or MCSP (FL-3). (B) MCSP fluorescence of total basal keratinocytes (Total) or 20% of cells with highest β1-integrin expression (β1-High) or 20% of cells with lowest β1-integrin expression (β1-Low). (C) β1 Integrin fluorescence of total basal keratinocytes (Total) or 20% of cells with highest MCSP expression (MCSP-High) or 20% of cells with lowest MCSP expression (MCSP-Low). (B,C) Dotted lines show the fluorescence of cells labelled with secondary antibody alone.
We conclude that selection on the basis of high β1 integrin and MCSP expression does not provide any further enrichment for clonogenic keratinocytes than selection for high β1 integrin levels alone. We also conclude that, given its trypsin sensitivity, MCSP does not provide a useful selectable cell surface marker for stem cell enrichment in vitro.

**MCSP cytoplasmic domain does not regulate terminal differentiation of human keratinocytes in vitro**

The cytoplasmic domain of MCSP is highly conserved between species (Fig. 5A) and mediates several functions of the proteoglycan, including modulation of integrin-mediated adhesion (Iida et al., 1995; Eisenmann et al., 1999; Fang et al., 1999; Stallcup and Dahlin-Huppe, 2001). In order to interfere with the function of MCSP in keratinocytes, we generated a chimeric construct consisting of the MCSP cytoplasmic domain fused to the extracellular and transmembrane domains of CD8, which is not endogenously expressed by keratinocytes, in the retroviral expression vector pBabepuro (pBp) (Fig. 5B). Populations of primary human keratinocytes or the human squamous cell carcinoma line SCC4 were then generated that expressed this construct (CD8/MCSP), full-length CD8 (CD8) or empty vector alone (Fig. 5CD). Expression of CD8 or CD8/MCSP had no effect on the levels of endogenous MCSP on the cell surface (Fig. 5D). There was no detectable shedding of the MCSP ectodomain (Nishiyama et al., 1995) in untransduced keratinocytes and SCC4 cells, nor cells expressing CD8 or CD8/MCSP (data not shown).

To investigate whether expression of CD8/MCSP influenced terminal differentiation, we used flow cytometry to establish the proportion of cells in confluent cultures that express involucrin, a precursor of the epidermal cornified envelope that is expressed by all suprabasal keratinocytes in culture (Jones and Watt, 1993). Expression of CD8/MCSP did not increase the proportion of involucrin-positive cells compared with either CD8 or vector alone controls, suggesting that expression of CD8/MCSP did not induce or suppress differentiation (Fig. 6A). Similar results were obtained with another marker of terminal differentiation, cornifin (Fujimoto et al., 1997) (data not shown).
We next investigated whether CD8/MCSP had any effect on the clonal growth of cultured human keratinocytes. Keratinocytes expressing CD8/MCSP, CD8 or empty vector were plated at clonal density onto mitotically inactivated feeders and cultured for 10-14 days. CD8/MCSP-expressing cells gave rise to an equivalent number of proliferative colonies and contained a similar proportion of abortive clones to the CD8 and vector-alone controls (Fig. 6B). Therefore, expression of CD8/MCSP had no effect on keratinocyte differentiation or proliferative capacity.

**CD8/MCSP decreases keratinocyte cohesiveness without affecting cell-ECM adhesion or motility**

When primary keratinocytes infected with CD8/MCSP were grown at clonal density, the morphology of the resulting colonies was strikingly different from that of colonies containing cells expressing full-length CD8 or the empty retroviral vector (Fig. 7A). CD8/MCSP-expressing colonies had an irregular shape, compared with the smooth, rounded borders of colonies formed by the controls. Individual cells within colonies often appeared to be trying to detach from the colonies, although cell-cell contact was usually maintained, and cells tended to have an elongated/polarized/motile appearance, compared with the typical keratinocyte 'cobblestone' morphology. These observations were confirmed by time-lapse microscopy of CD8/MCSP colonies; in addition, the time-lapse movies revealed that cells around the periphery of colonies were more motile and there was more movement within colonies (see Movies 1 and 2 at http://dev.biologists.org/supplemental/). The effects of CD8/MCSP were more obvious in small colonies and were less apparent upon confluence, although confluent cultures of CD8/MCSP-expressing keratinocytes frequently appeared to be disorganized (data not shown).

To investigate whether the altered colony morphology of keratinocytes expressing CD8/MCSP was due to altered cell/extracellular-matrix adhesion or motility, we analysed the behaviour of individual CD8/MCSP-expressing keratinocytes. Neither untransduced keratinocytes nor keratinocytes expressing CD8 or CD8/MCSP adhered to type-VI collagen, the major extracellular-matrix ligand for MCSP (Tillet et al., 1997; Tillet et al., 2002) (data not shown). No differences were observed in the proportion of cells that adhered to type-I collagen after 24 hours (data not shown) (Zhu et al., 1999). The motility of CD8/MCSP-expressing keratinocytes was not significantly different from CD8 and vector-alone controls when individual cells plated sparsely were examined (Fig. 7B).

**CD8/MCSP perturbs intercellular adhesion and the actin cytoskeleton, effects that can be mimicked by inhibiting Rho and are independent of ligation of the CD8 extracellular domain**

We next investigated whether cell-cell adhesion was disrupted by CD8/MCSP expression. Immunofluorescence analysis of...
Role of MCSP in epidermis cells at the edges of large keratinocyte colonies showed that both CD8/MCSP and CD8 were localized to actin-based plasma membrane structures such as protrusions and microvilli, and were concentrated at cell-cell contacts (Fig. 8A). CD8/MCSP colocalized with endogenous MCSP on the cell surface (data not shown). The appearance of cell-cell contacts was markedly different in CD8/MCSP-expressing cells compared with controls. Whereas control cells had well-defined, discrete borders, the junctions of CD8/MCSP-expressing cells appeared more diffuse because of CD8/MCSP-positive protrusions extending between adjacent cells (Fig. 8A).

Immunofluorescence staining with an anti-E-cadherin antibody revealed that adherens junctions were disorganized by CD8/MCSP. E-Cadherin was still localized to intercellular borders but the staining was more diffuse than in control colonies and E-cadherin was expressed on the CD8/MCSP-induced intercellular protrusions (Fig. 8B). The protrusions also stained with phalloidin, showing that they contained polymerized actin (Fig. 8C). Taken together, these observations suggest that CD8/MCSP disrupts E-cadherin-based adherens junctions, potentially via an effect on the cortical actin cytoskeleton.

The perturbation in E-cadherin and actin observed in keratinocytes expressing CD8/MCSP suggested that the chimera might modulate the activity of Rho GTPases (Etienne-Manneville and Hall, 2002). We examined this by incubating keratinocytes that expressed CD8 or CD8/MCSP with different pharmacological inhibitors and examining the effects on the distribution of E-cadherin (data not shown) and polymerized actin (Fig. 9A). We treated cells with the C3 exoenzyme from Clostridium botulinum, which inhibits the function of RhoA, RhoB and RhoC; the other Rho family proteins Rac-1 and Cdc42 are poor C3 substrates (Udagawa and McIntyre, 1996). We also used Y-27632, a specific inhibitor of the Rho-associated coiled-coil-forming protein serine/threonine kinase (ROCK) family of protein kinases (Narumiya et al., 2000). In addition, we treated cells with LY294002 to inhibit phosphatidylinositol 3-kinase, which is a downstream effector of Rac (Pullen and Thomas, 1997).

When keratinocytes transduced with CD8 were treated with C3 or Y-27632, the actin-based protrusions at cell-cell borders became more pronounced and the cells resembled CD8/MCSP-expressing keratinocytes that had not been exposed to the drugs (Fig. 9A). The effect of LY294002 on CD8-expressing cells was modest, with some increase in cortical actin assembly (Fig. 9A). C3 and Y-27632 did not overcome the effects of...
CD8/MCSP on E-cadherin and actin distribution but exacerbated them, whereas LY294002 had no obvious effect (Fig. 9A and data not shown). These experiments suggest that CD8/MCSP might inhibit Rho activity.

Ligation of NG2 with antibodies can trigger changes in the actin cytoskeleton (Fang et al., 1999; Majumdar et al., 2003), so we investigated the effect of ligating CD8/MCSP. We induced clustering of the CD8 extracellular domain by incubating transduced keratinocytes with mouse anti-CD8 followed by anti-mouse IgG (Fig. 9B). There was no detectable effect of inducing capping of the CD8 extracellular domain either in cells expressing CD8 or CD8/MCSP. Capped cells were compared with untreated cells (Fig. 9A) and cells treated either with anti-CD8 alone (Fig. 9B) or anti-mouse IgG alone (data not shown). We conclude that the effects of CD8/MCSP on the actin cytoskeleton are independent of ligand induced clustering.

**Role for MCSP in stem-cell cohesiveness**

To examine whether interference with endogenous MCSP had any effect on cell-cell adhesion, we cultured SCC4 cells in low-calcium medium and transferred them to standard medium for 2 hours. This treatment induces the assembly of adherens junctions and desmosomes, and the concentration of actin at cell-cell borders (Hodivala and Watt, 1994; Braga et al., 1995). As previously reported (Hodivala and Watt, 1994), anti-E-cadherin partially inhibited calcium-induced cell-cell adhesion and the accumulation of polymerized actin at cell-cell borders (Fig. 10A). The NG2 ectodomain (data not shown) or antibodies to MCSP (Fig. 10A) did not prevent cell-cell adhesion or actin reorganization. However, the combination of anti-MCSP and anti-E-cadherin perturbed these processes more than anti-E-cadherin alone (Fig. 10A).

To investigate the functional importance of the perturbed intercellular adhesiveness of keratinocytes expressing CD8/MCSP, we performed experiments in which the distribution of clones of keratinocytes expressing CD8/MCSP in confluent sheets of wild-type keratinocytes was examined (Fig. 10B). For this analysis, confluent keratinocyte sheets were reconstituted from CD8/MCSP or CD8-expressing keratinocytes cultured with an excess of unlabelled keratinocytes of the same strain and passage that expressed empty vector alone. Clones of keratinocytes expressing CD8/MCSP or CD8 growing amongst unlabelled neighbours were then detected using an anti-CD8 mAb and scored for cohesiveness (Fig. 10B,C).

Whereas most clones formed by CD8-expressing keratinocytes were cohesive with few detached cells, clones formed by CD8/MCSP-expressing keratinocytes were more dispersed (Fig. 10B). Single cells were frequently observed to have detached from the edges of CD8/MCSP-positive clones, whereas this was rarely observed in CD8-positive clones. No differences between the sizes of CD8- or CD8/MCSP-expressing clones were observed, consistent with the finding that CD8/MCSP did not affect keratinocyte differentiation or clonogenicity (Fig. 6).

All CD8- and CD8/MCSP-positive clones were scored for cohesiveness (Lowell et al., 2000; Lowell and Watt, 2001). Clones in which fewer than 30% of cells were detached from the main clone were scored as cohesive and clones in which greater than 30% of cells were not associated with the clone were scored as non-cohesive. Expression of CD8/MCSP strongly inhibited cohesiveness of clones, with 78.5% of CD8-expressing clones being cohesive compared with 26% of CD8/MCSP-expressing clones (Fig. 10C). We conclude that MCSP regulates keratinocyte cohesiveness by a mechanism that is independent of differentiation and motility.

**Discussion**

We have observed heterogeneous expression of MCSP in the basal layer of human IFE. The MCSP-positive keratinocytes co-expressed high levels of β1 integrins and were not actively cycling, characteristics that identify MCSP as a marker of the stem-cell compartment (Jones et al., 1995; Jensen et al., 1999). The combination of MCSP and high β1 integrin expression did not enrich for clonogenic cells more than high β1 integrin
Fig. 8. Effect of CD8/MCSP on intercellular adhesion. Colonies of primary human keratinocytes expressing CD8/MCSP, CD8 or vector alone were fixed, permeabilized and labelled with anti-CD8 (A), anti-E-cadherin (B) or TRITC-conjugated phalloidin (C) and viewed by confocal microscopy. Regions demarcated by boxes are shown at higher magnification on the right.

Fig. 9. Effects of pharmacological inhibitors (A) and antibody crosslinking (B) on the actin cytoskeleton of keratinocytes expressing CD8 or CD8/MCSP.
(A) Keratinocytes were incubated in medium alone (control) or supplemented with C3 toxin, Y-27632 or LY294002 to inhibit Rho, ROCK and phosphatidylinositol 3-kinase, respectively.
(B) Keratinocytes were incubated with OKT8 alone (cont) or OKT8 in combination with anti-mouse IgG (X link) to induce crosslinking of the CD8 extracellular domain. Cells were labelled with TRITC-conjugated phalloidin. Scale bar, 40 μm.
expression alone, and interference with MCSP function by overexpressing the cytoplasmic domain of MCSP had no effect on the proportion of terminally differentiating or clonogenic keratinocytes. However, CD8/MCSP-expressing clones were less cohesive than controls and this correlated with perturbation of E-cadherin and cortical actin at cell-cell borders, possibly via inhibition of Rho. Anti-MCSP enhanced the inhibitory effects of antibodies to E-cadherin on cell-cell adhesion. We therefore propose that one function of MCSP is to contribute to stem cell clustering by promoting cell-cell adhesion.

When the cytoplasmic domains of β integrin subunits or classical cadherins are coupled to an irrelevant extracellular domain, such as CD8, the resultant chimeras have a dominant negative effect on cell/extracellular-matrix adhesion (Chen et al., 1994; LaFlamme et al., 1994; Lukashev et al., 1994) or cell-cell adhesion (Kintner, 1992; Fujimori and Takeichi, 1993), respectively. Such constructs have been expressed in human epidermal keratinocytes via retroviral infection. A b1 integrin chimera reduces extracellular-matrix adhesion and activation of mitogen-activated protein kinase, thereby promoting exit from the stem-cell compartment (Zhu et al., 1999). A cadherin cytoplasmic domain chimera inhibits cell-cell adhesion (Zhu and Watt, 1996) but also stimulates differentiation by depleting the cytoplasmic pool of β-catenin available for signalling (Zhu and Watt, 1999). Based on these observations, we believe that CD8/MCSP acts as a gain-of-function mutation. Nevertheless, the high expression of MCSP in stem-cell clusters and the dispersion of cells expressing CD8/MCSP fit well with the model that CD8/MCSP has a dominant negative mechanism of action.

The adhesive function of NG2/MCSP has been attributed to extracellular-matrix adhesion and collagen types V and VI are known ligands (Tillet et al., 1997). Several cell surface proteoglycans, including MCSP, have been reported to modulate integrin function (Iida et al., 1995; Couchman and Woods, 1999; Eisenmann et al., 1999) by lateral association with integrins and other cell surface receptors within the plasma membrane (Woods and Couchman, 2000). NG2/MCSP collaborates with the α4β1 integrin in mediating adhesion and spreading on fibronectin via activation of the Rho-family GTPase Cdc42 (Iida et al., 1995; Eisenmann et al., 1999). NG2-mediated adhesion does not, however, depend on integrins, because introduction of NG2 into β1 integrin-negative cells promotes attachment to collagen types V and VI (Tillet et al., 2002). In the absence of β1 integrins, engagement of NG2 by collagen type VI triggers cell spreading and rearrangement of the actin cytoskeleton (Tillet et al., 2002). MCSP forms an association with membrane-type-3 matrix metalloproteinase, and this interaction might play a role in triggering invasion through extracellular matrix (Iida et al., 2001).

Although MCSP was expressed by those keratinocytes with the highest levels of β1 integrins, we did not obtain any evidence that MCSP-mediated keratinocyte/extracellular-
matrix adhesion. Keratinocytes do not express α4β1 integrin (Watt, 2002) and we did not see convincing localization of MCSP to focal adhesions. Keratinocytes did not adhere to type-VI collagen regardless of whether they expressed CD8/MCSP (data not shown), and CD8/MCSP had no effect on adhesion to type I collagen or on motility of individual cells. It is nevertheless interesting that FRAS1, the gene that is mutated in Fraser syndrome, has sequence similarity to NG2 and there is epidermal detachment from the basement membrane in Fraser syndrome patients (McGregor et al., 2003).

In addition to its extracellular-matrix adhesive functions, NG2 modulates cellular responses to growth factors, a common property of cell surface proteoglycans (Kresse and Schontherr, 2001). NG2 is required for the responsiveness of aortic smooth muscle cells to platelet-derived growth factor (PDGF) AA (Grako and Stallcup, 1995; Grako et al., 1999). NG2 and the PDGF α receptor are co-expressed in immature oligodendrocyte progenitors and antibodies to NG2 block their response to PDGF (Nishiyama et al., 1996). Basic fibroblast growth factor also binds NG2 (Goretzki et al., 1999). NG2 binds to plasminogen and to angiotatin, which contains plasminogen kringle domains; soluble NG2 enhances the activation of plasminogen by urokinase-type plasminogen activator and inhibits the antagonistic effect of angiotatin on proliferation of endothelial cells (Goretzki et al., 2000). In our experiments, there was no effect of CD8/MCSP on keratinocyte growth or terminal differentiation but this does not exclude a potential role for MCSP in modulating the local concentration of growth factors within the epidermis.

Although no role has previously been reported for NG2/MCSP in cell-cell adhesion, our data are consistent with the known association of NG2 with the actin cytoskeleton (e.g. Fang et al., 1999). The localization of MCSP and CD8/MCSP on keratinocytes is also in good agreement with an earlier report that NG2 is found on cell surface microspikes and microvilli (Garrigues et al., 1986). The cytoplasmic domain of NG2 binds a multiple-PDZ-domain-containing protein called MUPP1; MUPP1 might be involved in linking NG2 to actin filaments or to cytoplasmic signalling cascades (Barritt et al., 2000). NG2 also regulates Rho-dependent mechanisms in the trailing processes of motile cells (Stallcup and Dahlin-Huppe, 2001) and can signal via Rac (Majumdar et al., 2003). The effect of CD8/MCSP on the actin cytoskeleton of keratinocytes could be mimicked by inhibiting Rho or ROCK (Fig. 9A), in keeping with the conclusion that inhibition of Rho prevents adherens junction assembly in epithelial cells (Étienne-Manneville and Hall, 2002). A role for MCSP in intercellular adhesion is also plausible given that the central region of the MCSP extracellular domain has a series of repeats that show weak similarity to, and might have a similar structural role to, repeats in cadherin extracellular domains (Staub et al., 2002). CD8/MCSP perturbed cadherin-mediated adhesion, and the interaction of another chondroitin-sulphate proteoglycan (neurocan) with its receptor coordinatey inhibits both N-cadherin and β1 integrin-mediated adhesion (Li et al., 2000).

It is interesting that neurocan is proposed to play a role in preventing cell and neurite migration across boundaries (Li et al., 2000), given that we are proposing a similar function, stem-cell clustering, for MCSP in the epidermis.

The finding that MCSP expression was confined to the basal layer of the epidermis agrees with the observation that NG2 is downregulated with the onset of terminal differentiation in a range of cell types (Grako and Stallcup, 1995; Dawson et al., 2000). However, whereas MCSP is a marker for the mitotically inactive stem cell compartment in the epidermis, NG2 is expressed on mitotically active progenitor populations (the progeny of stem cells that are committed to differentiate) in the central nervous system. For example, NG2 is widely used as a marker for oligodendrocyte progenitors in the adult mammalian central nervous system (Dawson et al., 2000; Hartmann and Maurer, 2001). NG2/MCSP is not expressed by normal haematopoietic cells but is expressed on the surface of leukaemic blasts in certain childhood leukaemias (Behm et al., 1996; Smith et al., 1996). MCSP is expressed on melanoma but not normal melanocytes, and we have noted upregulation of MCSP expression in squamous cell carcinoma lines (J.L. and F.M.W., unpublished; see also Fig. 2E). The overall significance of MCSP expression in different cell types is, however, difficult to assess because of the diverse potential functions of the proteoglycan in adhesion and growth factor responsiveness.

In conclusion, MCSP expression is a new marker of stem cells in human IFE. It is not particularly useful for purifying stem cells from cultured epidermis because it is texpin sensitive and does not enrich for clonogenic cells any more than β1 integrins. Nevertheless, it does appear to be functionally important in promoting stem-cell clustering. NG2-null mice exhibit no gross phenotypic abnormalities (Grako et al., 1999), but it would be interesting to see whether the location of stem cells is altered in the epidermis. MCSP might contribute to the stem-cell microenvironment by influencing the accessibility of growth factors to keratinocytes and modulating cell/extracellular-matrix adhesion in ways that we have yet to uncover.

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References


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