Regulation of CD44v6-containing Isoforms during Proliferation of Normal and Malignant Epithelial Cells

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ABSTRACT

CD44 is a family of molecules involved in cell–cell and cell–matrix interactions. Various isoforms of CD44 arise by insertion of one or more of the variant exons into the common backbone shared by all forms of CD44. In this work, we studied the expression of CD44 and exon v6-containing CD44 isoforms (CD44v6) in several nonmalignant and malignant conditions and the possibilities for regulating the expression of CD44v6. In primary squamouscellular carcinomas of the head and neck, CD44 and CD44v6 were down-regulated in poorly differentiated tumors, whereas these molecules were uniformly expressed in the normal squamouscellular epithelium, in proliferating skin diseases, and in nonmalignant tumors. When CD44v6 expression of original tumors and that of squamouscellular carcinoma cell lines derived from them were compared, no CD44v6 up-regulation could be observed on in vitro growing cells. Moreover, several regulators were unable to up-regulate CD44v6 expression on cultured cell lines in vitro. When the same cell lines formed tumors after s.c. injection into severe combined immunodeficient mice, some of them up-regulated their CD44v6 expression. These data suggest that cell lines at certain differentiation stages can be induced to express CD44v6. Our results further indicate that CD44v6 positivity cannot be used as a universal indicator of tumor metastasis. Instead, the down-regulation of CD44v6 in squamouscellular tumors is a sign of malignant transformation of the epithelium.

INTRODUCTION

CD44 is a multifunctional glycoprotein involved in diverse cell–cell interactions. They include lymphocyte-endothelial cell binding (1–3), adhesion of cells to extracellular matrix proteins (4–7), lymphphopoi etosis, homotypic adhesion (8), T-cell activation and adhesion (9–14), cytokine release (15), and lateral movement of cells (16). CD44 is expressed in several cell types including leukocytes, erythrocytes, many types of epithelial cells, fibroblasts, smooth muscle cells, and glial cells of the central nervous system (17, 18).

Most hematopoietic cells, fibroblasts, and glial cells express a 90-kDa form of CD44, but lymphocytes also have a minor chondroitin sulfate-containing 180-kDa form (19). CD44 antigen in epithelial cell lines is considerably larger (140–160 kDa), and still larger forms up to 230 kDa have been described (3, 20, 21). These bigger isoforms of CD44 arise by alternative splicing that results in insertion of 1 or more of the 10 variant exons into the extracellular part of the 90-kDa constant form of the molecule (22).

Several CD44 isoforms play a role in the development and spread of malignancies. Specifically, CD44v6 is overexpressed in several tumors. In the rat, CD44v6 confers metastatic potential to a pancreatic adenocarcinoma cell line (23). In humans, expression of v6-containing variant proteins has been reported to be strongly associated with tumor development and metastatic potential of colorectal cancer, and it also seems to determine the length of survival in breast cancer (24, 25). It has even been suggested that the expression of certain CD44 splice variants is restricted to tumors and that the analysis of tissue or cell samples for CD44 variants by PCR could be used for cancer screening (26, 27).

We have observed earlier down-regulation of CD44v6 in SCC. In that small and heterogenous group containing both recurrent and primary carcinomas, it was not possible to correlate CD44v6 to any clinical parameter (28). The purpose of this study was to further analyze the clinical significance and the mechanisms of modulation of this particular isoform. Therefore, we investigated the expression of CD44v6 in epithelial cells and cell lines as well as in a well-defined group of 49 primary squamouscellular cancers of the head and neck and correlated it with several clinicopathological parameters and prognosis. To find out whether proliferation and/or migration of epithelial cells are connected to the loss of CD44 on SCC, several nonmalignant skin diseases and healing wounds were also analyzed. We also studied the effect of cell differentiation on CD44v6 expression in epithelial cells in vitro. These experiments were supplemented with in vivo studies using an animal model (SCID mice). In addition, the effect of UVB irradiation on epidermal CD44 expression of eight volunteers was analyzed. The results show that in contrast to many other cancer types, CD44v6 is down-regulated during malignant transformation of squamouscellular epithelium. Moreover, a short-term UVB exposure cannot cause this down-regulation, and it is not connected to proliferation of epithelial cells as such. Furthermore, regulation of CD44v6 expression on keratinocytes could only be induced under in vivo conditions.

MATERIALS AND METHODS

Patients. Forty-nine patients with histologically diagnosed SCC in the Turku University Central Hospital in 1988–1994 were studied. Thirty patients were men, and the median age was 64 (range, 25–93) years. All carcinomas were primary carcinomas of the head and neck. They were classified and graded into three grades according to the WHO classification (29). Clinical staging was done according to the International Union Against Cancer TNM classification (30). The median follow-up of patients alive was 25 months. Most patients were treated with preoperative radiotherapy of 60–65 Gy in 6–7 weeks followed by surgery. Surgery was considered as radical in 38 cases, palliative in 5 cases, and in 6 cases only a biopsy was taken. Radiotherapy was given to 44 patients and was considered to be radical in 42 cases. In 46 cases, the treatment given was considered as curative and in 3 cases as palliative.

Eight healthy volunteers, three of whom were men, were exposed to UVB irradiation (Corona Mini equipped with Philips TL 20 W, F 20T12 tubes; Esstål, Vårnamo, Sweden) on a 1-cm² square on the volar aspect of a forearm. The dosage was 1.5 standard erythemal doses (1.5 standard erythemal doses corresponding to 1.5 × the dose that produces in a nonexposed sun-sensitive white skin an erythema reaction). The skin-punch biopsies were taken from the unexposed normal skin and from the exposed area 2 and 7 days after irradiation.

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3 The abbreviations used are: SCC, squamouscellular carcinoma; SCID, severe combined immunodeficient; IL, interleukin; TGF, transforming growth factor; PMA, phorbol 12-myristate 13-acetate; mAb, monoclonal antibody.

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tion. The cutaneous reaction was scored using a visual scale: −-, no erythemal reaction; +/−, faint erythema with indefinite borders; +, moderate erythema with sharp borders; and ++, vivid erythema with edema or vesicles.

In addition, skin-punch biopsies were taken from patients suffering from the following benign skin diseases: lichen ruber planus (two patients), psoriasis (four patients), seborrheic keratosis (three patients), pemphigus (one patient), dermatitis (four patients), and papilloma (two patients).

The skin-punch specimens were also taken from the skin of one healthy volunteer and 3 and 8 days after the original biopsy at the healing edge of the wound. All tissue sampling and experimental protocols were approved by institutional human or animal research committees, and informed consent of the patients was always obtained.

Cells, Cell Lines, and Inducers. HaCaT, a spontaneously transformed human epithelial cell line from adult skin, which maintains full epidermal differentiation capacity was a kind gift from Professor N. E. Fusenig (German Cancer Research Center, Heidelberg, Germany; Ref. 31). HaCaT cells were cultured in RPMI 1640 supplemented with 10% FCS, 4 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml), and also in serum-free keratinocyte medium supplemented with either low (0.2 mM) or high (1.4 mM) Ca²⁺ (Life Technologies Ltd., Middlesex, England) and passaged using trypsin-EDTA (Boehringer Mannheim, Mannheim, Germany). For multilayered cultures of HaCaT, the cells were first seeded on collagen-coated Transwells (Costar, Cambridge, MA). After the confluence was reached, culturing was continued in an air-liquid interface for a minimum of 2 weeks in the presence of fibroblasts in the lower chamber. The cultures were grown in all different media presented above. Carcinoma cell lines were established from patients with cancer of the head and neck region as described previously (32, 33). All of the cancer lines express squamous cell-specific antigens, and, in addition, the UT-MUC-1 shows a mucopidermoid appearance (33, 34). Cells were maintained in DMEM containing 1% nonessential amino acids, 2 mM L-glutamine, penicillin, streptomycin, and 15% FCS. Hospital records of all of the patients from whom the cell lines were obtained were reviewed and the previous history, treatment, and follow-up records were summarized.

Tumor necrosis factor-α, IL-1, IL-3, IL-6, IFN-γ, TGF-β, PMA, and epidermal growth factor were used to study the induction of CD44v6 expression on the surface of HaCaT cells, and, in addition, four carcinoma cell lines were incubated with IL-1 and TGF-β. All of these cytotoxins were products of Genzyme (Cambridge, MA) and PMA was obtained from Sigma (St. Louis, MO). Tunicamycin, β-d-xyloside, and sodium chloride (Sigma) were used as glycosylation and sulfation inhibitors. Retinol (Sigma) and methotrexate (Orion-Farmos, Turku, Finland) were used to modify keratinocyte differentiation. To study the effect of extracellular matrix components on the expression of CD44v6, HaCaT cells and four carcinoma cell lines were also grown on laminin, fibronectin, heparan sulfate, chondroitin sulfate, and collagen I (all from Sigma) coated chamber slides (Nunc, Naperville, IL). In addition, HaCaT cells were also cultured on Matrigel (Becton Dickinson, Bedford, MA). Concentrations of and incubation periods with these regulators are listed in Table 1.

Animals. SCID mice were obtained from Bombolgaita Breeding and Research Center Ltd. (Bommice, Denmark).

Antibodies. The production and specificity of anti-CD44 mAbs Hermes-3 and Var 3.1 have been described elsewhere (1, 28). Hermes-3 recognizes an epitope in the proximal extracellular part of the constant region of CD44 (35), thus detecting all forms of CD44. The mAb Var 3.1 is directed against the synthetic peptide representing a 16-amino acid sequence from the exon v6 of the human variant CD44. mAb HB116 against HLA-ABC was from American Type Culture Collection (Rockville, MD). The mAb 3G6, a mouse antibody that recognizes laminin 1 and mAb GB3 against laminin-5 (kalinin) have been obtained from ICN Biomedicals (Costa Mesa, CA) and mAb 1345 against type VII collagen was a product of Chemicon (Temecula, CA). Antibody used to recognize laminin-1 and mAb GB3 against laminin-5 (kalinin) have been described elsewhere (36, 37).

Immunoperoxidase Staining. The distribution of the standard and variant v6 forms of CD44 was determined using acetone-fixed frozen sections and indirect immunoperoxidase staining as described (28). Antibody staining intensity was scored on a semiquantitative scale as −−, −, +, ++, and ++++, where + represents weak staining intensity, ++ moderate staining intensity, and ++++ corresponds to the staining intensity of normal squamoepithelium present in many samples. Staining intensity was scored independently by three readers and in ~10% of the borderline cases with discordant scoring a consensus was sought. All staining classifications were done without knowledge of the results of the other analyses or clinical data. Statistical analyses were done using a BMDP computer program (BMDP Statistical Software, Department of Biomatics, University of California Press, Los Angeles, CA). Frequency tables were analyzed using the χ² test or Fisher's exact test. Cumulative survival was estimated with the product-limit method, and comparison of cumulative survival between the groups was done using the log rank test. All Ps are two-tailed.

Immunofluorescence Staining. HaCaT and cancer cell lines grown on chamber slides (tissue culture chambers; Nunc) were stained with and without acetone fixation using an indirect immunofluorescence method as described (28).

Injection of HaCaT Cells and Cancer Cell Lines into SCID Mice. To study the expression of CD44v6 in vivo, we grew keratinocyte and tumor nodules in SCID mice. HaCaT cells and two cancer cell lines were detached from the culture flasks by trypsinization as described above and resuspended in RPMI 1640 without supplements. Aliquots (0.2 ml) containing 5 × 10⁶ cells were injected s.c. into the back of SCID mice. During the succeeding 2 weeks nodules developed. At days 2, 5, 7, 14, and 21 the mice were sacrificed. The nodules were excised, frozen in liquid nitrogen, and processed for immunoperoxidase staining as described above.

RESULTS

Down-Regulation of CD44 Isoforms in Malignant Transformation. CD44 forms detected with Hermes-3 antibody (CD44v6) and with Var 3.1 (CD44v6) were strongly expressed in normal squamoepithelium. Expression patterns of CD44st and CD44v6 resembled each other. In the skin, all epidermal layers except the keratinized upper layer expressed CD44st and CD44v6. The most striking difference was in the dermis. Dermal fibroblasts strongly expressed CD44st, but were practically devoid of CD44v6. Instead, hair follicles and sweat glands expressed both CD44st and CD44v6.

Only 3 of 49 SCCs showed weak expression of CD44st. The rest were moderately or strongly CD44st positive (Fig. 1, a and b). Expression of CD44st correlated with the histological grade of SCCs studied. Strong CD44st expression was associated with a high histological grade of differentiation (grade 1, Table 2). Only 23% of grade

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Regulators used to induce CD44v6 expression in HaCaT cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Regulator</strong></td>
<td><strong>Dose</strong></td>
</tr>
<tr>
<td>Cytokines/growth factors</td>
<td></td>
</tr>
<tr>
<td>IL-1</td>
<td>40 and 80 units/ml</td>
</tr>
<tr>
<td>IL-3</td>
<td>500 and 1000 units/ml</td>
</tr>
<tr>
<td>IL-6</td>
<td>500 and 1000 units/ml</td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
</tr>
<tr>
<td>Tumor necrosis factor</td>
<td></td>
</tr>
<tr>
<td>α</td>
<td>400 units/ml</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1000 units/ml</td>
</tr>
<tr>
<td>TGF-β</td>
<td>1 ng/ml</td>
</tr>
<tr>
<td>Epidermal growth factor</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td>PMA</td>
<td>50 ng/ml</td>
</tr>
<tr>
<td>Regulators of differentiation</td>
<td></td>
</tr>
<tr>
<td>Methotrexate</td>
<td>1 and 5 μM</td>
</tr>
<tr>
<td>Retinol</td>
<td>1 and 5 μg/ml</td>
</tr>
<tr>
<td>Calcium</td>
<td>1 and 5 mM</td>
</tr>
<tr>
<td>Glycosylation/sulfation inhibitors</td>
<td></td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>10 μg/ml</td>
</tr>
<tr>
<td>β-d-Xyloside</td>
<td>1 and 5 mM</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 and 10 mM</td>
</tr>
<tr>
<td>Extracellular matrix components</td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td>20 μg/ml</td>
</tr>
<tr>
<td>Laminin</td>
<td>20 μg/ml</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>Chondroitin sulfate</td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>Collagen 1</td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>Matrigel</td>
<td>100 μg/ml</td>
</tr>
</tbody>
</table>

*Contains predominantly entactin, collagen IV, laminin, proteoglycans, heparan sulfate, and nidogen (Ref. 57, and the manufacturer’s information).
Table 2 Expression of CD44st and histological grade of SCCs of the head and neck

<table>
<thead>
<tr>
<th>Grade</th>
<th>n</th>
<th>Negative (0%)</th>
<th>Weak (0%)</th>
<th>Moderate (39%)</th>
<th>Strong (61%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>0</td>
<td>1</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

CD44st staining intensity

2 or 3 carcinomas had strong CD44st expression as compared with 61% of the grade 1 carcinomas (P = 0.007). An association between CD44st expression and tumor size was also found. Only 6 of 22 (27%) T3–4 tumors showed strong CD44st expression as compared with 14 of the 27 (52%) T1–2 carcinomas (P = 0.08). No association (P > 0.1) was found between CD44st expression and gender, age at diagnosis (≤ median versus > median), WHO performance status (0 or 1 versus 2 or 3) or the cervical nodal status (N0 versus N+).

Expression of CD44v6 was weak in 18 carcinomas, and practically no CD44v6 expression was detected in 19 carcinomas. Examples are shown in Fig. 1, c and d. Low CD44v6 expression was associated with low expression of CD44st. Only 2 of the 19 (11%) cancers that were entirely negative in staining for CD44v6 strongly expressed CD44st, whereas 18 (60%) of the cancers that showed weak to strong expression of CD44v6 expressed CD44st strongly (P = 0.0006). Like CD44st, low CD44v6 expression was associated with poorly/moderately differentiated carcinomas (Table 3). Only 5 of 23 (22%) well-differentiated (grade 1) carcinomas were entirely negative for CD44v6 as compared with 14 of 26 (54%) moderately/poorly differentiated carcinomas (P = 0.02). No significant association was found between CD44v6 expression and tumor size, gender, age, cervical nodal status, or performance status. Neither the expression of CD44st nor the expression of CD44v6 was associated with survival.

Expression of CD44v6 Is Not Induced on Cancer Cell Lines in Vitro. Expression of CD44v6 in cancer cell lines was determined to explore the possibility that a suppressive factor(s) avoidable in vitro conditions down-regulates CD44v6 in vivo. The original tumor sample was available from four of eight tumors from which the lines had been propagated, and, thus, were used for direct comparison. Patient characteristics, tumor site, and histological classification as well as the expression of CD44v6 of the cell lines are presented in Table 4. One carcinoma cell line of eight expressed moderate amounts of CD44v6 on its surface, two were totally negative, and five were weakly or very weakly positive. Six carcinoma cell lines were intracellularly positive. There was no up-regulation of surface CD44v6 expression in the cells.

Table 3 Correlation of CD44v6 expression and histological grade of tumors

<table>
<thead>
<tr>
<th>Grade</th>
<th>n</th>
<th>Negative (0%)</th>
<th>Weak (0%)</th>
<th>Moderate (13%)</th>
<th>Strong (0%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>5 (22%)</td>
<td>10 (44%)</td>
<td>6 (26%)</td>
<td>2 (9%)</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>7 (39%)</td>
<td>7 (39%)</td>
<td>2 (11%)</td>
<td>2 (11%)</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>7 (88%)</td>
<td>1 (13%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>
Table 4 Characteristics of the patients and specimens used to establish the carcinoma cell lines, and CD44v6 expression of the original tumor and the cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Donor</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Primary tumor location</th>
<th>TNM classification</th>
<th>Specimen site</th>
<th>Type of lesion</th>
<th>Grade</th>
<th>CD44v6 cytoplasmic</th>
<th>CD44v6 surface</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: UT-SCC-12A</td>
<td>F</td>
<td>82</td>
<td></td>
<td>Nasal skin</td>
<td>T2N0M0</td>
<td>Nose</td>
<td>Primary</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2: UT-SCC-16A</td>
<td>F</td>
<td>77</td>
<td></td>
<td>Tongue</td>
<td>T2N0M0</td>
<td>Tongue</td>
<td>Primary</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3: UT-MUC-M</td>
<td>F</td>
<td>74</td>
<td></td>
<td>Hard palate</td>
<td>T2N0M0</td>
<td>Neck</td>
<td>Metastasis</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>33</td>
</tr>
<tr>
<td>4: UT-SCC-2</td>
<td>F</td>
<td>75</td>
<td></td>
<td>Gingival ulcer</td>
<td>T2N1M0</td>
<td>Gingiva</td>
<td>Primary</td>
<td>2</td>
<td>ND</td>
<td>+/-</td>
<td>58</td>
</tr>
<tr>
<td>5: UT-SCC-5</td>
<td>M</td>
<td>60</td>
<td></td>
<td>Base of the mouth</td>
<td>T2N0M0</td>
<td>Floor of mouth</td>
<td>Primary</td>
<td>2</td>
<td>ND</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>6: UT-SCC-7</td>
<td>M</td>
<td>68</td>
<td></td>
<td>Temporal skin</td>
<td>T2N0M0</td>
<td>Neck</td>
<td>Metastasis</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>60</td>
</tr>
<tr>
<td>7: UT-SCC-4</td>
<td>F</td>
<td>43</td>
<td></td>
<td>Hypopharynx and larynx</td>
<td>T3N1M0</td>
<td>Larynx</td>
<td>Recurrence</td>
<td>2</td>
<td>ND</td>
<td>++</td>
<td>61</td>
</tr>
<tr>
<td>8: UT-SCC-11</td>
<td>M</td>
<td>58</td>
<td></td>
<td>Vocal cord</td>
<td>T1N0M1</td>
<td>Larynx</td>
<td>Primary</td>
<td>2</td>
<td>ND</td>
<td>+++</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Lack of effect of UVB on the expression of CD44st and CD44v6 in normal human skin. Expression of CD44st in unexposed skin (a) and after 2 days (b) of the exposure. Staining of the unexposed skin sample with a class-matched negative control antibody (c). Expression of CD44v6 in unexposed skin (d) and after 2 days (e) of the exposure. E, epidermis, ×250.

Growing in culture, thus arguing against the existence of a suppressive factor(s) functioning in vivo.

**Effect of Short-Term UVB Irradiation on the Expression of CD44st and CD44v6.** To find out whether a short-term exposure to UV light is able to cause down-regulation of CD44st and CD44v6, we irradiated the skin of eight volunteers and investigated the expression of CD44st and CD44v6 after the exposure. The UVB dosage used (1.5 standard erythemal doses) corresponds to approximately 30 min of exposure to noon sunlight in Central Europe in spring. The response of the individuals to irradiation varied greatly, from mild redness to blistering of the skin (+/-, three of eight; +, three of eight; and ++, two of eight individuals 2 days after the exposure). Despite the variations in the severity of the skin reaction, UVB irradiation did not cause any marked changes in the expression of CD44st or CD44v6. In the UVB-irradiated skin, CD44st and CD44v6 were localized in the same layers of epidermis as in the normal skin, and the localization was similar 2 and 7 days after the exposure. Examples of the expression of CD44st 2 days and CD44v6 7 days after the exposure are shown in Fig. 2.

**Benign Lesions Express Normal Levels of CD44st and CD44v6.** CD44st and CD44v6 expression were also studied in a variety of skin diseases to find out whether proliferation as such is the cause of down-regulation. Keratinocytes in inflammatory lesions expressed similar levels of CD44st and CD44v6 as keratinocytes in the normal skin. Moreover, also benign tumors showed normal levels of both CD44st and CD44v6 expression, indicating that tumorigenesis is not
sufficient to cause a decrease in expression of these molecules (Fig. 3). Since proliferation could not cause down-regulation of CD44st and CD44v6, we also analyzed the expression of these molecules during the wound healing. A slight decrease in the expression of both CD44st and CD44v6 was seen in the leading edge of the migrating epithelium (Fig. 4), suggesting that CD44 may not be critical for active movement of keratinocytes.

**CD44v6 Expression Cannot Be Up-Regulated in Vitro.** To further study the regulation of CD44v6 expression, a keratinocyte cell line, HaCat, was used. These cells brightly express CD44st at intercellular junctions, whereas the leading edges remain CD44st dull (Fig. 5). HaCaT also faintly expresses CD44v6 and the staining is predominantly intracellular (Fig. 5 and Ref. 28). None of the several cytokines and mediators studied (see Table I) up-regulated the expression of the CD44v6 epitope recognized by Var 3.1 in HaCat cultured as a monolayer (data not shown). Methotrexate, retinol, and calcium (different concentrations), which modified the differentiation and morphology of the keratinocytes, did not alter the expression of CD44v6 (data not shown). No increase in CD44v6 expression was observed in the presence of glycosylation and sulfation inhibitors (tunicamycin, β-d-xyloside, and sodium chlorate), indicating that glycosylation or sulfation are not responsible for the lack of the positive staining with mAb Var 3.1. Also direct contacts between HaCaT cells and the components of the extracellular matrix (Table 1) were unable to up-regulate CD44v6 expression (data not shown). These results indicate that regulation of CD44v6 expression on keratinocytes is not under the control of the factors tested. Because HaCaT cells are known to maintain capacity for full epidermal differentiation, we also grew them in organotypic cultures. During the culturing, HaCaT cells formed multilayer organization. They brightly expressed CD44st, but this three-dimensional culture could not induce expression of CD44v6 (Fig. 6). Lack of in vitro up-regulation of CD44v6 was not a phenomenon restricted to HaCat cells, since four carcinoma cell lines (lines 2, 3, 4, and 5; Table 4) cultured in the presence of IL-1 and

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**Fig. 3.** Expression of CD44v6 remains at the normal level in benign skin diseases. A psoriatic skin lesion is stained with antibodies against CD44st (a) CD44v6 (b) and with a negative control antibody. Expression of CD44v6 in a papilloma sample is presented in d. E, epidermis. X100.

**Fig. 4.** Expression of CD44st and CD44v6 in an 8-day-old wound. a. The basement membrane is shown by anti-collagen IV staining. Open arrow, beginning of the intact basement membrane. b. The leading edge loses the prominent surface expression of CD44v6. (Compare the staining of the edge with that of normal epidermis in the upper right corner.) c. Expression of CD44st is also decreased in the migrating edge. d. Staining with the negative control antibody. In all panels the arrows indicate the migrating edge. E, epidermis; GT, granulation tissue. X100.
TGF-β and on different extracellular matrix components did not show up-regulation of CD44v6 (data not shown).

**CD44v6 Can Be Up-Regulated in Vivo.** To study the regulation of expression of CD44st and CD44v6 in vivo, HaCaT cells as a single-cell suspension were injected s.c. into SCID mice. In vivo, the cells reaggregated and formed small round nests with multilayered sheaths of keratinocytes. The cyst contains basement membrane components, since laminin-5 and α5β1 integrin were detectable after 2 days and laminin-1, type IV collagen, and type VII collagen after 5 days of injection. From day 2 on the cysts contained differentiated stratified epidermal cells which by day 21 had filled the central cavity. All cell layers of the cyst wall of 2–7-day-old cysts were positive for CD44st. On day 14, CD44st started to disappear from the uppermost layers next to the cyst cavity and 21-day-old cysts contained only the narrow CD44st-positive line above the basement membrane. The staining pattern of CD44v6 resembled that of the standard form in 7-day-old cysts with a well-differentiated multilayered cyst wall, but the intensity was clearly weaker (Fig. 7, a–d). Thus, in contrast to in vitro culture conditions, in vivo the HaCaT cells are able to up-regulate their CD44v6 expression. To further test whether this in vivo up-regulation is an artifact generated in the SCID mouse system or whether the ability to induce CD44v6 expression is linked to the capacity to differentiate, we also injected two carcinoma cell lines into SCID mice (nine animals/group). Cell line 2 (Table 4), although originally derived from a CD44v6-negative grade 3 carcinoma, behaved much like HaCaT cells. It grew rather slowly as small nodules (diameter 3–5 mm) up to 7 days after the injection, whereafter the nodules started to involute. Tumor cells in nodules expressed a moderate level of CD44v6. In contrast, cell line 4 (Table 4) grew rapidly at the injection sites, and the animals had to be sacrificed after 14 days of injection due to massive growth. In these tumors, only the most differentiated cells expressed CD44v6, whereas the majority of the cells remained CD44v6 negative (Fig. 7, e–g), indicating that the SCID microenvironment does not uniformly up-regulate CD44v6 expression of all cells, and the differentiation stage of the cell may determine whether the synthesis of CD44v6 can be turned on.

**DISCUSSION**

The most important findings of this work were that, in contrast to many other malignancies, expression of CD44v6 was down-regulated in SCCs of the head and neck. Moreover, expression of CD44 molecules as a group was also diminished, especially in more undifferentiated carcinomas. This down-regulation was not due to suppressive factors of the body, because in general the tumor cells did not show up-regulation of CD44 when grown in culture, and it could not be experimentally caused by a short-term exposure to UV light. Furthermore, up-regulation of CD44v6 expression could not be induced in cultured cell lines using several extracellular matrix components and soluble mediators. However, when transplanted into SCID mice, certain cell lines expressed CD44v6 at higher levels than in monolayer or multilayer cultures in vitro. These findings suggest that high expression of CD44st and CD44v6 is a typical feature of normal squamous epithelial cells, and it is down-regulated during malignant transformation.

The function of different isoforms of CD44 in normal squamous
epithelium is still poorly understood. The concentration of CD44 along cellular boundaries in vivo suggests that at least certain isoforms mediate homotypic cell adhesion within the epithelium as described earlier for T cells (8). This idea is also strengthened by the finding on the localization of CD44 on HaCaT cells and keratinocytes during the wound healing. When the HaCaT cells are grown in monolayer cultures, the most intensive staining for CD44 is detected in intercellular junctions, whereas at the leading edges markedly less positivity is seen. Moreover, the migrating epithelium in the healing wound shows diminished expression of CD44. The components of the extracellular matrix, hyaluronan, fibronectin, and collagen are known to serve as ligands for CD44 (4–6). Detailed studies of the capacity of different isoforms of CD44 to bind to hyaluronan that is an abundant component of the intercellular matrix of squamous epithelium have been performed in several laboratories (38–41). Although the results are not unequivocal, it seems obvious that different isoforms display different hyaluronan-binding properties. For example, the standard hematopoietic form of CD44 without any variant exons binds efficiently to hyaluronan, whereas the forms containing variant exon 3 show negligible binding (40). Adhesion is also controlled by the activation status of the CD44-expressing cell, and particular carbohydrate modifications of CD44 may also be decisive in the hyaluronan binding (41–44). Thus, adhesive functions of CD44 in cell–cell and cell–matrix interactions may be important in maintaining the normal architecture of the epithelium, and down-regulation of CD44 may offer the cell a possibility to detach and invade.
Down-regulation or total loss of CD44 may also have consequences to cell proliferation. CD44, especially the form containing exon v3, has been shown to bind growth factor (45). If binding to CD44 is envisioned as a regulator of interaction between the growth factor and its principal receptor, loss of CD44 allows enhanced binding between the growth factor and its receptor. This may then lead to accelerated cell proliferation. This hypothesis is supported by the present finding that squamous cell carcinomas showing diminished expression of CD44 are more often poorly differentiated and proliferate more rapidly than the well-differentiated ones expressing marked levels of CD44. Also in line with this is the behavior of the cell lines transplanted into SCID mice. The cell lines expressing moderate levels of CD44v6 involved, whereas the negative one (>95% cells negative) grew rapidly. Consistent with this idea is also the fact that rapidly proliferating highly aggressive non-Hodgkin’s lymphomas are often CD44 negative (46).

Behavior of CD44v6 in SCCs contrasts with that observed in many other tumor types. The first report of Günthert et al. (23) on CD44v6 presented evidence for the importance of a v6-containing isoform of CD44 in the metastasis formation of rat adenocarcinoma. Since then several reports on the role of CD44v6 in human malignancies have been published emphasizing the central role of CD44v6 in the spread of malignancies. For example, in colorectal and breast carcinomas, CD44v6 expression has been associated with tumor growth and metastasis formation, and in non-Hodgkin’s lymphomas CD44v6 expression has been associated with poor overall survival (24, 47, 48). On the other hand, contradictory results have also been presented (49, 50). There are only a few reports on malignant cell types which lose CD44v6 expression. In addition to our initial report containing staining data only on a few samples of SCC (28), other reports of CD44 down-regulation were published while we were preparing this article. Hudson et al. (51) found reduced expression of CD44 isoforms in a series of 13 oral SCCs and the loss was most common in poorly differentiated tumors. Comparable findings were also reported by Seelentag et al. (52). In addition, decreased expression of CD44v6 was found to be associated with poor prognosis in laryngeal SCC (53). Moreover, transitional cell carcinomas of the bladder have been shown to down-regulate CD44v6 (54–56). Based on the results obtained with various tumor materials gathered in different laboratories around the world, it is obvious that CD44v6 positivity cannot be used as a universal indicator of tumor metastasis.

Changes in the expression of CD44 are dependent on the cell type and perhaps also on the local microenvironment of the cell undergoing malignant transformation. Moreover, the genomic diversity of CD44 allows the synthesis of different variant isoforms containing v6 alone or in combination with one or more of the nine other variant exons of the extracellular part of CD44. The neighboring exons may well modify the function of the molecule, and therefore, detection of v6 as such does not necessarily indicate the same CD44-dependent behavior for every cell expressing v6. Therefore, generalizations of the role of CD44v6 in the metastatic spread of cancer have to be made with caution. However, among a specialized group of cancers such as squamouscancer, CD44v6 can be used as a diagnostic tool. In this case down-regulation or total disappearance of CD44v6 is an indicator of malignant transformation of squamous epithelial cells.

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Regulation of CD44v6-containing Isoforms during Proliferation of Normal and Malignant Epithelial Cells

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