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Simone Seiter · Wolfgang Tilgen · Karin Herrmann
Dirk Schadendorf · Erik Patzelt · Peter Möller
Margot Zöller

Expression of CD44 splice variants in human skin and epidermal tumours

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Abstract Splice variants of the adhesion molecule CD44 (CD44v) are important in the lymphatic spread of rat carcinoma cells. In several human tumours expression of CD44v correlates with tumour progression. However, little is known about the physiological functions of distinct variant exons. Here we report on the immunohistological evaluation of CD44 expression in normal human skin and epidermal tumours which do not metastasise, or do so vary rarely. Frozen tissues were stained with a panel of monoclonal antibodies, recognizing epitopes of the CD44 standard isoform, as well as of variant exons v5, v6, v7, v7-v8 and v10. Stratum basale and spinosum as well as the root shaft of hairs reacted strongly with the whole panel of anti-CD44 antibodies. Stratum corneum, acinar cells of sebaceous and eccrine sweat glands stained with anti-CD44v5, anti-CD44v6 and anti-CD44v7, but not with anti-CD44v10, the latter recognizing the "epithelial isoform" (CD44v8–v10) of CD44. Ductal cells of glands and apocrine glands did not express CD44v. Compared with its expression in normal human skin, CD44v expression was reduced in basal cell carcinoma and squamous cell carcinoma of the skin. This was particularly true of CD44v10. The expression of CD44v in normal skin and dermal appendages indicates that not all combinations of variant exons are involved in tumour progression. Since the epithelial isoform is particularly downregulated in basal cell carcinoma and squamous cell carcinoma of the skin, it is unlikely that exons v8–v10 play a role in tumour progression. Rather, they may be of functional importance in maintenance of the epidermal structure.

Key words CD44 variant isoforms · Skin · Basal cell carcinoma · Spindle cell carcinoma

S. Seiter · W. Tilgen
Department of Dermatology, University of Heidelberg, Heidelberg, Germany

K. Herrmann · M. Zöller (✉)
Department of Tumour Progression and Immune Defense, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany
Fax: (49) 6221-42 47 60

D. Schadendorf
Department of Dermatology, Rudolf Virchow Hospital Berlin, Berlin, Germany

E. Patzelt
Bender GmbH, Vienna, Austria

P. Möller
Institute of Pathology, University of Heidelberg, Heidelberg, Germany

Introduction

CD44 comprises a family of glycoproteins which vary by glycosylation and protein structure [5, 9, 29, 35, 39, 41]. As demonstrated in Figure 1, the latter is mainly due to alternative splicing, whereby up to ten additional, so called variant exons can be inserted into the membrane proximal extracellular domain of the standard or haematopoetic form of the molecule (CD44s) [45, 52]. These variant exons can be expressed individually or in a variety of combinations [16, 20, 25–28, 46, 52], and the same cell can express more than one combination of variant exons [2, 6, 10, 11, 14, 16, 42, 48]. Some of these combinations are found, in particular, in specific organs or tissues, like the epithelial form, which contains the exons v8–v10, and the keratinocyte form, which contains the exons v3–v10 [4, 8, 20, 34, 49, 54]. Both the exons of the standard part of the molecule and the variant exons are heavily glycosylated: N-linked and O-linked glycosylations as well as chondroitin sulphate side chains have been described [4, 5, 21, 30, 35]. Furthermore, the region of the variant exons is highly hydrophilic and may thus be involved in specific ligand interactions [15, 21]. Finally, since alternative splicing of CD44 is stringently regulated, it can be assumed that the different exons or combinations of exons exert defined and divergent functions [15, 22].

Special interest in CD44 variant isoforms (CD44v) arose when it was demonstrated in a rat pancreatic carcinoma model that the expression of splice variants, in par-

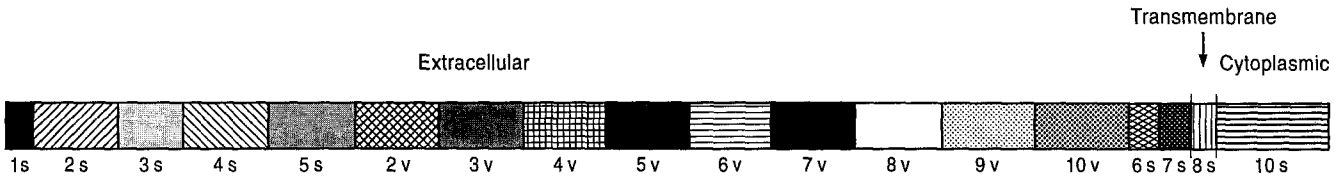

Fig. 1 Schematic representation of CD44 isoforms. Variations in the protein structure of CD44 are mainly due to the insertion of up to ten (mouse and rat) or 9 (human) so-called variant exons between exon 5s and 6s of the haematopoetic form of the molecule. Exon 8s comprises the transmembrane, exon 9s or 10s the cytoplasmic region

ticular exons v4–v7, initiated lymphatic spread of locally growing tumours [16]. In view of the high degree of interspecies conservation of the CD44 molecules [3, 46], it was hoped that CD44v may represent a unique and universal metastasis marker for human malignancies. In fact, in the majority of studies published in the meantime, expression of CD44v was observed in human malignancies, [reviewed in 12, 17, 37, 50, 60]. However, while expression of CD44v correlated with progression and/or dedifferentiation in some tumours [18, 31, 33, 55], less stringent correlations have been noted in others [32, 40, 43, 48], and even downregulation/loss of CD44v has been described [43].

Here we report on the expression of CD44v in epidermal tumours against the background of the phenotype of normal skin and dermal appendages. Most layers of the human epidermis as well as most of the appendages expressed CD44 splice variants. Expression of CD44v in epidermal malignancies was unaltered or reduced.

Materials and methods

The monoclonal antibodies SFF2 (anti-CD44s, IgG1), VFF8 (anti-CD44v5, IgG1), VFF7 (anti-CD44v6, IgG1), VFF9 (anti-CD44v7, IgG1), VFF17 (anti-CD44v7-v8, IgG2b) and VFF16 (anti-CD44v10, IgG1) were used as first antibodies. SFF2, VFF7, VFF8, VFF9, VFF16 and VFF17 were derived from BALB/c mice immunized with GST-CD44v3–v10 vusion protein. The exon-specificity was identified by binding to GST-fusion proteins, which contained single CD44 variant exons. The recognized epitopes were defined by a competition enzyme-linked immunosolvent assay using sets of overlapping peptides as competitors. The antibodies were further selected for the recognition of CD44v on tumour lines, which were known to express CD44v to guarantee that the specific epitopes were not hidden by glycosylation. In addition, Scatchard plot analysis revealed that the antibodies exhibited similar binding constants (a manuscript describing these antibodies in detail is in preparation). Culture supernatants were purified by passage over ProteinA- or ProteinG-Sepharose 4B. Eluates were dialyzed, concentrated and filter sterilized. A biotinylated polyclonal sheep antibody to mouse IgG (Sigma) was used as second antibody. First and second antibodies were diluted in phosphate buffered saline and titrated on positive and negative control cells. Final working concentrations for the first antibodies were in the range of 2.5–5 μg/ml; the second antibody was diluted 1:150.

Tissues were obtained from surgical specimens and biopsies. Fresh tissue was snap-frozen in liquid nitrogen. Serial sections of about 1 cm^2 were cut in 4–6 μm thick sections, mounted on gelatine coated slides and air dried overnight. They were fixed in acetone for 10 min at –20° C and stored at –20° C or immunostained immediately. Typing of epithelial tumours was carried out according to the guidelines of the WHO. The collection contained 37 basal cell carcinomas (5 solid, 8 solid multicentric, 8 sclerosing, 4 nodulo-ulcerative, 4 adenoid cystic, 4 pigmented and 4 superficial) and 24 epidermal squamous cell carcinomas. By differentiation, 6 squamous cell carcinoma were grade 1, 11 were grade 2, 4 were grade 3 and 3 were grade 4. None of the patients with squamous cell carcinoma had developed metastases at the time of excision of the primary tumour. Also, during the post-surgery observation periods of 12–36 months neither local recurrence nor metastatic settlement were observed.

For immunohistochemistry [7], fixed and dried sections were incubated for 1 h at room temperature with the first antibody. Slides were washed intensively and incubated at room temperature for 30 min with the second, biotinylated anti-mouse Ig antibody. After washing sections were incubated with a horseradish-peroxidase-conjugated streptavidin complex. 3-Amino-9-ethylcarbazole (Sigma) was used as substrate for the enzyme. The peroxidase reaction resulted in an intense red precipitate. The sections were faintly counterstained with Mayer's haematoxylin, air dried and mounted.

For negative controls the staining of each sample was performed without applying the primary antibody or by using an antibody of irrelevant specificity. With the exception of the reaction of the granulocytes due to their endogenous peroxidase, which had not been blocked, no staining was observed. The results were evaluated according to the following score system: high intensity of staining, ++; distinct staining, +; weak staining, ±; heterogenous staining, +>– (more stained cells), +/– (equal numbers of stained and unstained cells), +<– (more unstained cells).

Results

Using immunohistochemistry and a panel of monoclonal antibodies which recognize epitopes on CD44s, on exon v5, v6, v7, the transition of exon v7 or v8 and exon v10, we observed that in human skin predominantly CD44v are expressed (Table 1). With the exception of the stratum corneum all epidermal layers expressed at least the variant exons v5, v6, v7, v8 and v10. The intensity of staining for individual epitopes showed marked variations (Fig. 2). While the epitope on the standard domain stained very intensely, staining of variant exons was generally weaker. Furthermore, there was a grading of the staining (the intensity decreased from the basal towards the apical layers). Exon v7 in particular, was hardly detectable in the upper layers of the stratum spinosum. The external and internal root shaft (lower and upper part) of the hair stained essentially like the stratum basale and stratum spinosum. Expression of CD44v in the different glands was more restricted. In the sebaceous glands the basal region stained distinctly with anti-CD44v5 and anti-CD44v6, but only weakly with anti-CD44v7, anti-CD44v7-v8 and anti-CD44v10. Ductal and acinar cells stained weakly with anti-CD44v5, anti-CD44v7-v8 and anti-CD44v10. Acinar and ductal cells of the eccrine sweat glands stained with anti-CD44v5, anti-CD44v6

Table 1 Expression of CD44 isoforms on normal skin and dermal appendages (*v* variant, *s* standard)

Tissue type	Expression of CD44v isoforms[a]					
	CD44s	v5	v6	v7	v7-8	v10
Epidermis						
Keratinocytes						
Stratum basale	++	++	++	++	++	++
Stratum suprabasale	++	++	++	++	+	++
Stratum spinosum	++	++	++	+[b]	+	+
Stratum corneum	–	–	–	–	–	–
Hair						
Shaft						
External root	++	++	++	±	–	+
Internal root	++	++	++	+	+	+
Corticular substance/cuticle	++	–	–	–	–	–
Cortex/outer layer	–	–	–	–	–	–
Sebaceous glands						
Acinar cells	++	+	+	±	±	±
Ductal cells	++	–	–	–	+	+
Basal cuboidal cells						
Monolayer	++	++	++	+	+	+
Stratified	++	++	++	–	+	±
Sweat glands						
Eccrine glands						
Acinar cells	++	++	+	–	+	+>–
Ductal cells	++	++	+	–	–	+>–
Apocrine glands						
Acinar cells	++	–	+<–[b]	–	+<–[b]	–
Ductal cells	++	–	–	–	–	–
Myoepithelial cells	++	–	–	–	–	–

[a] Score system: high intensity of staining, ++; distinct staining, +; weak staining, ±; heterogeneous staining, +>– (more stained cells), +/– (equal numbers of stained and unstained cells), +<– (more unstained cells)
[b] Only basal cells are stained

and anti-CD44v10. Yet, in both instances not all cells were positive. In the apocrine glands, ductal and myoepithelial cells did not react with any of the anti-CD44v antibodies; only a minority of the acinar cells in the basal region stained with anti-CD44v6 and anti-CD44v7-v8. Other constituents of the skin, like vessel walls, smooth muscles, nerves and melanocytes (as revealed by double staining with a melanocyte specific antibody, not shown) did not react with any of the variant exon specific antibodies.

To evaluate whether malignant transformation is accompanied by changes in expression of the metastasis-associated CD44v comprising exon v6 or of the epithelial isoform comprising exons v8–v10, sections of 37 basal cell carcinomas and 24 epidermal squamous cell carcinomas were stained with the same panel of anti-CD44 monoclonal antibodies.

Expression of CD44v in both tumour types mostly followed the expression of CD44v as observed in the normal counterpart cells (Fig. 3). There was, however, a tendency towards loss of expression. All squamous cell carcinomas expressed exons v5 and v6. Anti-CD44v7 stained only subpopulations of tumour cells in the specimens of 16 of 24 patients. Likewise, anti-CD44v7-v8 as well as anti-CD44v10 stained only part of the tumour cells in 21 specimens. The ratio of stained to unstained cells varied between the individual tumours. It is worthwhile noting that staining was mostly confined to the cells at the inner wall towards the horny pearls rather than to the outer rim of the tumour (Fig. 4). Reduced expression of CD44v did not correlate with the grade of differentiation.

When compared with squamous cell carcinomas, expression of CD44v was even more markedly reduced in basal cell carcinomas (Fig. 4). Only 2 of the 37 specimens stained uniformly with anti-CD44v5, anti-CD44v6, anti-CDv7, anti-CD44v7-v8 and anti-CD44v10. With 15 and 18 specimens, respectively, staining was heterogeneous for exons v5 and v6. Anti-CD44v7 stained all cells in 9 tumours; the number of stained cells in the remaining 28 samples was lower than in anti-CD44v5 and anti-CD44v6 treated probes. Exons v7-v8 and v10 could not be detected in 3 and 5 basal cell carcinomas, respectively. These exons were frequently expressed in only minority of tumour cells in the remaining specimens. Unlike the squamous cell carcinomas, where stained and unstained cells were diffusely dispersed, the basal cell carcinomas consisted mostly of defined clusters of tumour cells, which either expressed or did not express CD44v. There was no obvious correlation of these features with the histological subtype.

Discussion

In the rat, lymphatic spread of carcinoma cells is essentially linked with the expression of CD44v [16, 24, 60], which under physiological conditions are expressed only

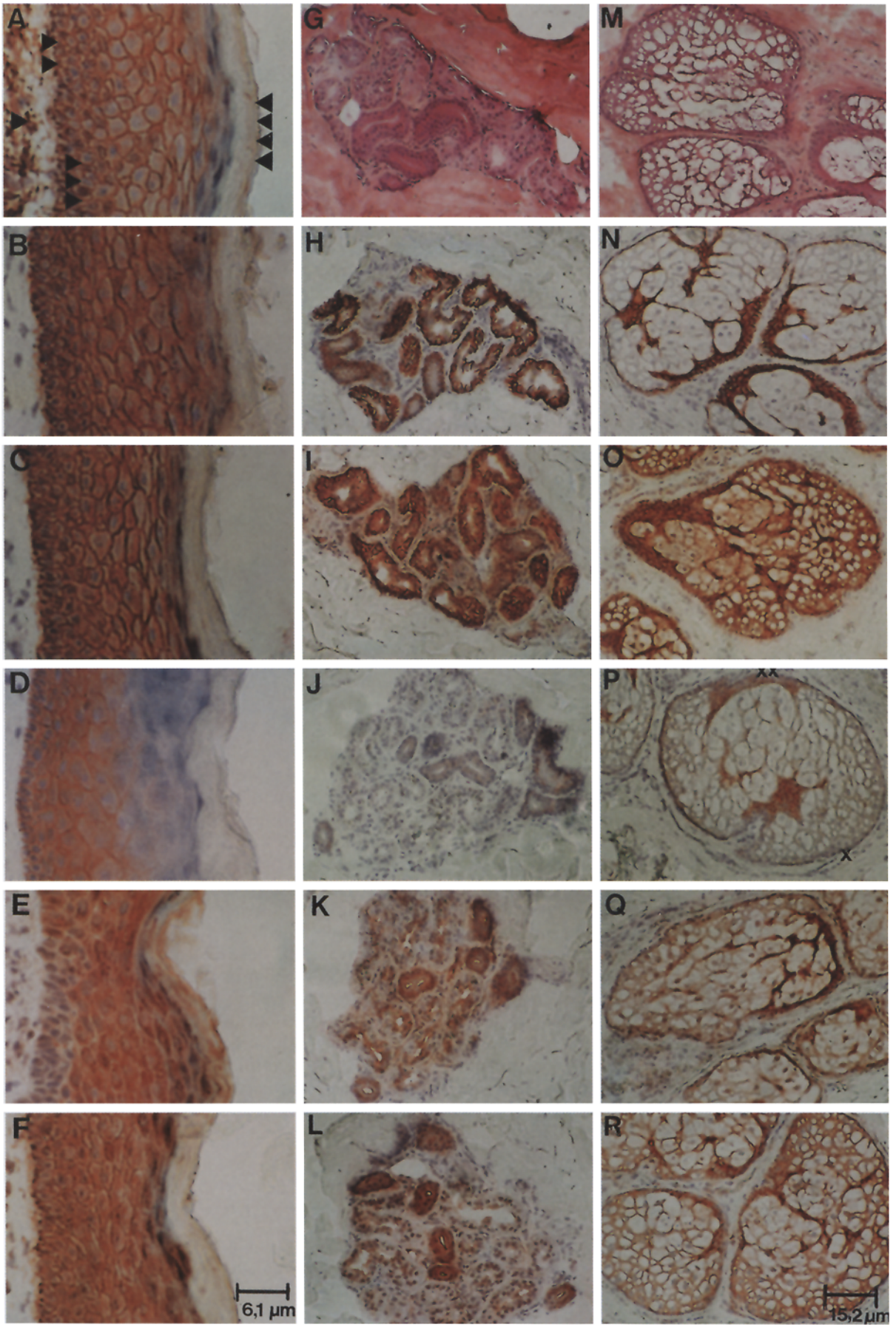
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Fig. 2A–R Expression of CD44 variant exons (v) in normal skin and appendages. Sections (4–6 μm) of normal skin were stained with anti-CD44s (**A**), anti-CD44v6 (**B**), anti-CD44v5 (**C**), anti-CD44v7 (**D**), anti-CD44v10 (**E**) and anti-CD44v7-8 (**F**). Stratum basale (▼▼) and stratum spinosum (▼▼▼) stained with all antibodies, although exon v7, v8 and v10 reached the upper layers of the stratum spinosum as compared to exon v5 and v6; stratum corneum (▼▼▼▼) stained only with anti-CD44v8 and anti-CD44v10; the subcutis (▼) only stained with anti-CD44s. (The few stained cells in the subcutis are in fact epidermal cells, which were dislodged by unevenness of the cutting level). *Bar* **A–F** 1 cm=6.1 μm. Staining of sweat gland (eccrine) (**G–L**) and of a sebaceous gland (**M–R**) with haematoxylin and eosin (**G, M**); monolayer x, stratified xx), anti-CD44v6 (**H, N**), anti-CD44v5 (**I, O**), anti-CD44v7 (**J, P**), anti-CD44v10 (**K, Q**) and anti-CD44v7-8 (**L, R**) revealed expression of distinct epitopes of CD44v in particular on acinar cells. *Bar* **G–R** 1 cm=15.2 μm

transiently and only in a few defined tissues [1, 56]. Considering these expression profiles, the question arose whether CD44v may represent an unifying metastasis marker in humans. This, however, is not the case. First, CD44v are more abundantly expressed in the human [13, 36, 51], especially in skin and other squamous epithelia [4, 8, 20, 34, 49, 54]. Second, tumour progression is not accompanied by de novo expression of CD44v [32, 40, 43, 48]: in the case of epidermal tumours, our data suggest that their locally invasive growth may even go in parallel with downregulation of defined CD44v. Interestingly, it is just the epithelial isoform comprising the exons v8–v10 which was partially lost in epidermal-derived tumours.

The family of CD44 molecules is supposed to fulfill a large variety of functions, whereby individual exons or combination of exons may fulfill very specialized tasks [reviewed in 15, 22, 59, 60]. Since the presumed relation between CD44 isoforms and functions is still elusive, an analysis of CD44v patterns in defined tissues and tissue layers could provide first evidence for potential functions of individual exon combinations as well as for their interrelationship. From our analysis of the skin, two interesting observations should be addressed: first, the pattern of CD44 isoform expression changes during differentiation; second, the epithelial isoform is (partially) lost in basal and epidermal squamous cell carcinomas.

CD44s and CD44v are abundantly expressed in the human epidermis. Expression of CD44s has been described in detail by Wang et al. [53]. Our findings are in line with this report, except that our anti-CD44s monoclonal antibody stained also myoepithelial cells of the apocrine glands. Expression of CD44v has also been described. In 1991, a so called keratinocyte type, comprising the exons v3–v10 and an epithelial isoform with exons v8–v10 were described at the RNA level [4, 8, 20, 34, 49, 54]. Using immunohistochemisty and in vivo labelling procedures we noted that in the rat exon v6 is abundantly expressed on the basal layer of the epidermis [56]. Terpe et al. [51] and Mackay et al. [36] screened human tissues for the expression of CD44v4, -v6 and -v9. In the study of Fox et al. [13] additional antibodies specific for CD44v3 and -v8/v9 were included and confirmed the findings at the mRNA level that in human skin the basal layers express CD44v abundantly. We here describe in detail expression of CD44v5, -v6, -v7, v7/v8 and -v10 in human skin and particularly in skin appendages and show that strong expression of the epithelial isoform containing the exons v8–v10 appears restricted to strata basale and suprabasale of the epidermis. Antibodies specific for the exons v5 and v6, however, also stained the hair shaft brightly, the basal cuboidal cells of sebaceous glands and acinar as well as ductal cells of eccrine glands. it should be mentined that expression profiles in acinar cells of sebaceous glands, which sometimes stain with anti- CD44v5 and anti- CD44v7, but not with anti- CD44v6, are difficult to interpret. Although analysis of tumour lines has revealed examples of the ex-

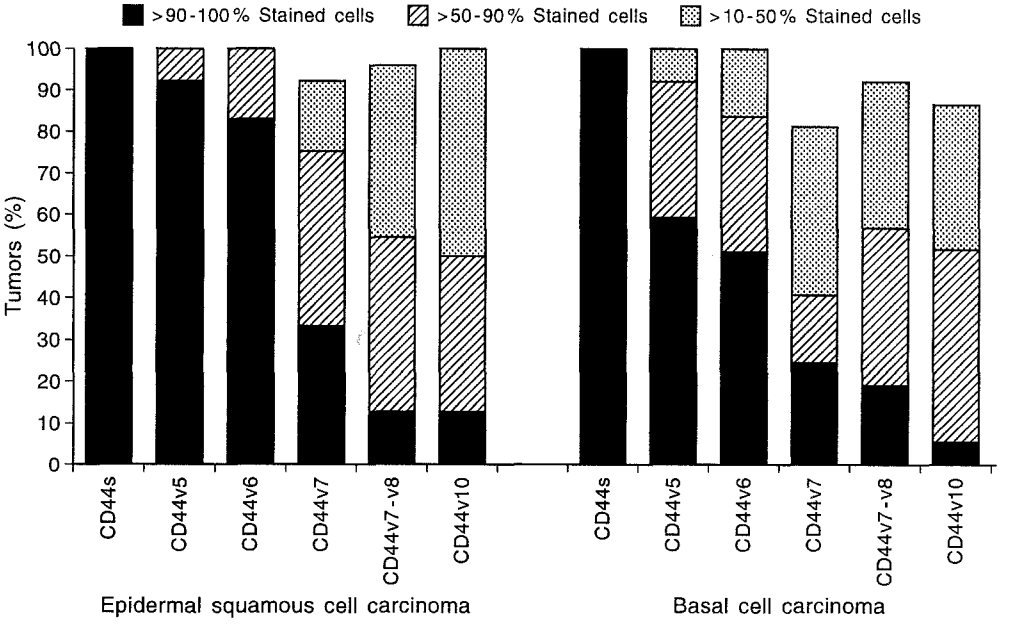

Fig. 3 Semiquantitation of expression of CD44v in basal cell and epidermal squamous cell carcinomas. Sections (4–6 μm) of basal (37) and epidermal squamous (24) cell carcinomas were stained with the panel of anti-CD44 monoclonal antibodies described above. All tumours were analysed semiquantitatively, grouped according to the estimated percentage of stained cells: few, <50% stained cells; most: 50%–90% stained cells; all, >90% stained cells. The percentages of tumours in the categories "few", "most" and "all cells" stained are shown

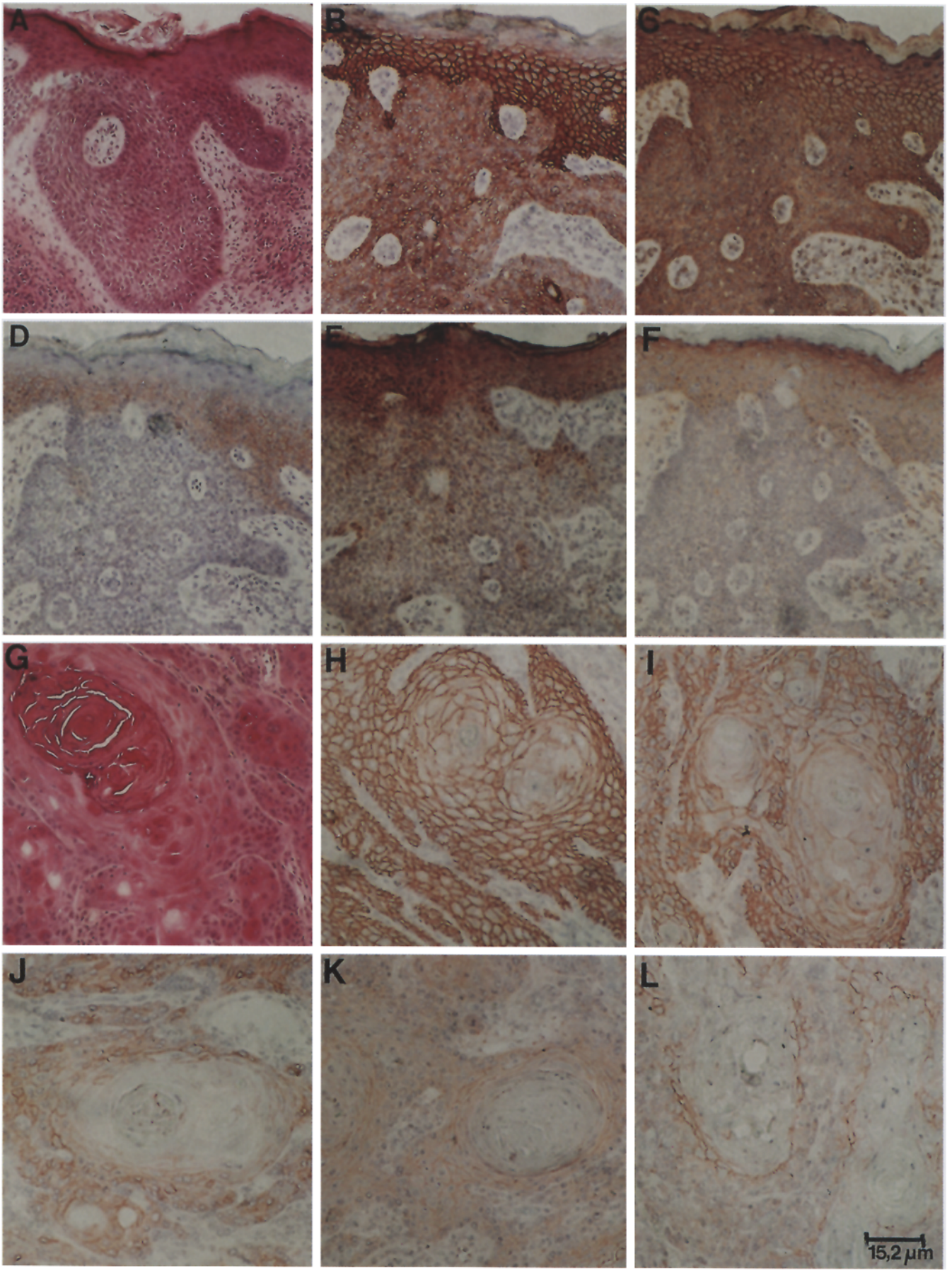
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Fig. 4A–L Expression of CD44v in epidermal squamous cell and basal cell carcinomas. Sections (4–6 μm) of a basal cell carcinoma (**A–F**) and a well-differentiated squamous cell carcinoma (**G–L**) were stained with haematoxylin and eosin (**A, G**), anti-CD44v6 (**B, H**), anti-CD44v5 (**C, I**), anti-CD44v7 (**D, J**), anti-CD44v10 (**E, K**) and anti–CD44v7-8 (**F, L**). Expression of exon v7 and the transition of exon v7-v8 was partially lost in both tumours. Expression of exon v10 was restricted to the inner zone of epidermal squamous cell carcinomas. Basal cell carcinomas displayed a heterogeneous staining pattern with antibodies specific for CD44v. *Bar* 1 cm=15.2 μm

pression of single variant exons, there are others where individual epitopes were hidden due to variations in the glycosylation and concomitant conformational changes. The latter explanation appears more likely, since CD44 is heavily glycosylated and even anti-CD44 monoclonal antibodies, which recognize overlapping epitopes can reveal rather different staining patterns. Finally apparent cytoplasmic staining was frequently observed which may be indicative of a high rate of CD44 synthesis. However, unequivocal distinction between surface and cytoplasmic staining is difficult in tissue sections.

Despite the careful analysis of CD44 variant exon expression in skin and skin appendages, it is not possible to draw conclusions on the CD44 exon combinations from surface staining. Since the various layers of the epidermis revealed distinct staining patterns, Western blots as well as Northern blots or amplification of cDNA reverse bromsciphan-polymerase chain reaction (RT-PCR) and Southern blotting with exon-specific probes cannot be expected to answer this question. In fact, we noted several signals in Western blots as well as in Southern blots after RT-PCR (data not shown). According to the size it is likely that at least CD44v containing v3–v10, v4–v7 as well as v8–v10 are present. Whether the same cell/cell layer or different cells/cell layers express these isoforms is unknown.

Expression of CD44v, mostly CD44v6, has been shown to correlate with tumour progression. Since basal cell carcinomas do not metastasize and none of the tested squamous cell carcinomas of the skin had developed metastases, one might have expected that expression of CD44v6 is unaltered. This was, indeed, the case. Furthermore, the loss of exons v5–v7 in the upper layers of the epidermis, which contains non-dividing cells, as well as the maintenance of expression of CD44v5 and CD44v6 in the tumour cells supports our working hypothesis that the exons v4–v7 are involved in the transduction of signals initiating cell proliferation [58, 59]. The assumption is corroborated by the following observations: these exons are expressed de novo during clonal expansion of lymphocytes [1]; transgenic mice, which express CD44v4–v7 on T lymphocytes shown an expanded and accelerated response towards nominal antigen [38]; proliferating progenitor cells in the bone marrow also express the exons v5 and v6 (and v9), but not the epithelial isoform (unpublished).

The functional activities of exons v8–v10 are unknown. Since CD44v8–v10 is the predominant isoform of the human epidermis [4, 8, 20, 34, 49, 54], it was of particular interest to explore expression of these exons in non- or rarely metastasizing tumours of the skin. Basal cell and spindle cell carcinomas which infiltrate the dermis lost these variant exons partially. We did not, however, observe a downregulation of CD44s, which has been described recently for alopecia areata [44], and for basal cell carcinomas [57]. Furthermore, during revision of this manuscript, another report was published concerned on expression of CD44s, CD44v4, CD44v6 and CD44v9 in primary skin tumours and metastasis [19]. Guttinger et al. described upregulation of CD44v in hyperproliferative disorders of the skin, but downregulation in primary skin tumours and metastasis. This finding is only partially in line with our observations of a selective downregulation of the epithelial isoform. The discrepancies between Yasaka et al. [57] and Guttinger et al. [19] may be due to the use of different antibodies and, as described above, masking of distinct epitopes by conformational changes. The notion of the partial loss of CD44v8–v10 on epidermal tumours suggest that this particular isoform may be required for the maintenance of epidermal structure and/or for integration/penetration into the coherent epidermal tissue. The findings that expression of the epithelial isoform is lost upon invasive growth of squamous cell carcinoma and particularly in metastases derived thereof [23] that the CD44v8–v10 isoform is most strongly expressed in skin metastases of malignant melanoma [47] and that skin infiltrating lymphocytes are CD44v10-positive are in agreement with this working hypothesis.

Taken together, the diversity of CD44v expression in the various layers of the skin, in skin appendages and in epidermal carcinomas thus provided the first experimental evidence for divergent functions of distinct variant isoforms. The requirement of CD44v8–v10 or CD44v10 for homing and integration into the epidermal tissue could well be of clinical importance in wound healing, allergic skin reactions and cutaneous metastasis formation. This should now be confirmed in functional studies with CD44v8–v10- or CD44v10-cDNA transfected lines.

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