Activated Human Lymphocytes and Aggressive Non-Hodgkin's Lymphomas Express a Homologue of the Rat Metastasis-associated Variant of CD44

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Summary

A recently described splice variant of CD44 expressed in metastasizing cell lines of rat tumors, has been shown to confer metastatic potential to nonmetastasizing rat pancreatic carcinoma and sarcoma cell lines. Using antibodies raised against a bacterial fusion protein encoded by variant CD44 sequences, we have explored the expression of variant CD44 glycoproteins on human lymphoid cells and tissues and on non-Hodgkin's lymphomas. Normal lymphohematopoietic cells express barely detectable low levels of variant CD44 glycoproteins, whereas T lymphocytes, upon activation by mitogen or antigen, transiently upregulate expression of specific CD44 variant glycoproteins. The reaction pattern of various antibodies indicates that these CD44 variants contain the domain encoded by exon v6, which is part of the variant that in the rat confers metastatic capability. It is interesting that overexpression of v6 was also found in several aggressive, but not low-grade, non-Hodgkin's lymphomas.

Tumor metastasis is the principle cause of death for cancer patients. A subset of parental tumor cells acquire these metastatic properties, presumably through a series of genetic alterations (1, 2). During the metastatic process, tumor cells detach from the primary tumor, migrate into the extracellular matrix (ECM)[2] and invade adjacent structures including blood and lymph vessels. Via lymph or blood, the tumor cells are subsequently transported to sites of metastatic outgrowth where they engage endothelium and the ECM. This process of tumor dissemination bears resemblance to that of lymphocyte trafficking. Both involve highly mobile cells, cellular interactions with and migration via ECM and endothelium, and cellular transport via blood and/or lymph. Moreover, cell adhesion receptors are believed to be critically important in both processes.

CD44, a heterogenous family of molecules with putative functions in cell–cell and cell–matrix adhesion, has recently been linked to tumor dissemination in several systems including human non-Hodgkin's lymphomas (NHL) (3–6), melanomas (7), and rat adenocarcinomas (8). In the rat system, highly metastatic pancreatic and mammary carcinoma cell lines were found to express splice variants of the CD44 glycoprotein (8). These variants differ from the standard CD44 molecule in that they contain additional peptide domains inserted into the extracellular portion of the transmembrane protein (8, 9). Some of these variant CD44 proteins have been shown to be causally involved in tumor metastasis formation. Coinjection of variant-specific mAb with the metastasizing cells led to retardation or even complete blockage of metastatic spread in vivo (10). Moreover, overexpression of the metastasis-specific CD44 variants in nonmetastasizing tumor cell lines led to lymphogenic metastatic spread (8). The major CD44 isoform on lymphocytes is the smallest standard molecule (CD44s). In the rat, lymphocyte activation increases the frequency of a (v6 exon containing) larger isoform which appears to be important for the immune response (11). Splice variants containing v6 are also expressed on several normal human epithelia and on carcinoma lines from lung, breast, and colon (9, 12). Such variants are also found in colorectal carcinomas and their metastases (12).

The similarity between tumor spread and lymphocyte

[1] The first three authors contributed equally to this paper.

[2] *Abbreviations used in this paper:* ECM, extracellular matrix; NHL, non-Hodgkin's lymphoma; TCGF, T cell growth factor; WF, International Working Formulation.

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trafficking and the putative role of CD44 in both these processes, raises the question whether CD44 splice variants expressed by lymphocytes might be involved in the migration of normal and malignant lymphocytes. In the present study, we have therefore explored the expression of CD44 variant glycoproteins on normal human lymphocytes and malignant lymphomas. Whereas expression of CD44 variants on normal lymphohematopoietic cells and tissues is very low, T cell activation by mitogen or antigen leads to a strong transient upregulation of CD44 variant expression. It is interesting that CD44 variants, particularly homologues of variants that confer metastatic behavior in the rat, are also expressed in aggressive malignant lymphomas.

Materials and Methods

Cloning of pGEX Fusion Proteins. The whole variant region of the HPKII type CD44v (9, and see Fig. 1) was cloned from human keratinocyte cDNA via PCR amplification. The two PCR primers 5'-CAGGCTGGGAGCCAAATGAAGAAAATG-3', positions 25–52, and 5'-TGATAAGGAACGATTGACATTAGAGTTGGA-3', positions 1013–984 of the LCLC97 variant region as described in Hofmann et al. (9), included an EcoRI recognition site, which was used to clone the PCR product directly into the pGEX-2T vector (13). The resulting construct (pGEX CD44v HPKII, v3–v10) codes for a fusion protein protein of ~70 kD.

To obtain subclones of the variant region that could later be used for affinity purification or Western blot analysis, DI (v3), DII/III (v5, v6), and DIII (v6, v7) comprising fragments (see Fig. 1) were cloned using appropriate restriction sites. Fusion protein DI contains the CD44 sequence described by Stamenkovic et al. (14) from position 744 to position 142 of variant CD44 described by Hofmann et al. (9), fusion protein DII/III contains the variant sequence from position 290 to 460, and fusion protein DIII the variant sequence from position 378 to 638 (9). The DI and DIII comprising fragments were cloned into the pGEX vector system, and the DII/III fragment was cloned into the pATH-vector (15).

Development of Polyclonal and Monoclonal Abs. To obtain polyclonal Abs against variant CD44, New Zealand White rabbits were immunized according to standard procedures. In brief, 200 μg of affinity-purified fusion protein derived from pGEX CD44v HPKII (v3–10) was injected subcutaneously together with 400 μl Freund's incomplete adjuvant four times within 9 wk. To remove Abs directed against the glutathione transferase part of the fusion protein, the pGEX-2T glutathione transferase fragment was coupled to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology, Uppsala, Sweden). The serum was applied three consecutive times to the column, the flow-through was collected and loaded onto affinity columns to which CD44v HPKII (v3–10) was coupled. Specific Abs were eluted with 100 mM glycine pH 2.5.

To obtain mAb, female BALB/c mice were immunized with the affinity-purified fusion protein derived from pGEX CD44v HPKII (exons v3–10) described above. Spleen cells of an animal with a high Ab titer were fused to P3X63Ag8.653 myeloma cells using polyethylene glycol 4000; hybridomas were selected in HAT medium (16, 17). Determination of serum Ab titers and screening of hybridomas were performed by ELISA. Briefly, the assay plates were coated with the fusion protein, incubated with serial dilutions of serum samples of hybridoma supernatants, and specific Abs were detected with peroxidase-coupled Abs to mouse Ig. Hybridomas reactive with glutathione transferase were eliminated. The remaining Abs were further characterized in ELISA using fusion proteins of the variable domains DI (exon v3), DII/III (exons v5, v6), and DIII (exons v6, v7), respectively. The reactivity of the Abs with normal human skin keratinocytes was determined by immunohistochemistry. For detection of standard CD44, we used mAb NKI-P1 (18).

Western Blot Analysis. Bacterial fusion proteins were taken up in gel sample buffer, boiled, and resolved on 10% SDS polyacrylamide (19). Proteins were transferred to polyvinylidene difluoride membranes (Millipore GmbH, Eschborn, Germany) using a transblot apparatus (BioRad Laboratories, Cambridge, MA). Nonspecific interactions were blocked with PBS containing 10% dry milk. Subsequently, the membranes were incubated at room temperature with the Abs, followed by alkaline phosphatase–conjugated goat anti-rabbit IgG (Amersham International, Amersham, Bucks, UK), for 1 h each. After each individual Ab incubation, the membranes were washed with PBS containing 0.3% Tween 20 (Sigma Immunochemicals, St. Louis, MO). Signals were developed with the enhanced chemoluminescent system (Amersham International).

Tissues. Normal and pathological tissues were selected from the files of the Department of Pathology, Academic Medical Center, University of Amsterdam, and tested for expression of CD44 and variant CD44, employing the mono- and polyclonal Abs described above. All normal tissues were obtained as a corollary of standard surgical procedures. NHL were classified according to the International Working Formulation (WF) (20). By their expression of T and B lineage antigens they were divided into T lineage and B lineage.

Immunohistochemistry. Immunoperoxidase staining was performed as described previously (12). In brief, cryostat sections were fixed in acetone for 10 min, washed in PBS, and preincubated with normal goat serum (10% in PBS). After preincubation, the sections were incubated with the primary Ab for 1 h. Before incubating with the secondary biotinylated Ab for 30 min (anti-mouse and anti-rabbit $F(ab)_2$; Dako Corp., Carpinteria, CA), endogenous peroxidases were blocked with 0.3% H_2O_2 in methanol. All Abs were titrated to give optimal staining results. For detection, a streptavidin–biotin–peroxidase complex (Dako Corp.) was used. After incubation for 30 min, the sections were incubated in 3,3-amino-9-ethyl carbazole (Sigma Immunochemicals) for 10–20 min.

Cell Isolation. PBMC from normal donors were isolated by Ficoll-Isopaque density gradient centrifugation. RBCs were taken from the Ficoll pellet and washed extensively in RPMI 1640 medium. Granulocytes were isolated from the Ficoll pellet by two rounds of RBC lysis with hypotonic ammonium chloride. PBMC were further purified in a lymphocyte (PBL) and monocyte fraction by monocyte adherence on plastic petri dishes (Falcon Plastics, Oxnard, CA) for 1 h at 37°C. The monocytes were harvested with a rubber policeman. Purity of PBLs, monocytes, and granulocytes was >95% as determined by FACS® analysis (Becton Dickinson & Co., Mountain View, CA) for CD45, CD14, and CD15 expression. For isolation of tonsil lymphocytes, freshly obtained tonsillar tissue was dissected free from surface epithelium and minced with scissors. Mononuclear cells were obtained by Ficoll-Isopaque density gradient centrifugation and monocytes were depleted by adherence on plastic petri dishes.

Cell Lines and Cell Cultures. The cell lines used were: CEM, HSB-2, and Jurkat, T lymphoblastoid cell lines/T-ALLs; JVM-2, B-PLL; U937, myelomonocytic line; and HaCat, spontaneously immortalized keratinocytes. T cells were activated by culturing PBMC in the presence of PMA (10 ng/ml), with the anti-CD3 mAb OKT3 (0.01%; Ortho Diagnostic Systems Inc., Raritan, NJ) and 5% T cell growth factor (TCGF), or with the anti-CD3 mAb

16A9 (Dr. R. A. W. van Lier, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), immobilized on plastic (ascites 1:1000, coated at 4°C). TCGF was produced as previously described (21).

Lymphoid cell lines and T cells were cultured in RPMI 1640 medium with 25 mM Hepes buffer (Gibco, Grand Island, NY), whereas HaCat was grown in DMEM (Gibco). All media were supplemented with 1 mM glutamine, 10% (vol/vol) heat-inactivated FCS (Hyclone Laboratories Inc., Logan, UT), 100 U/ml natrium penicillin G (Gist Brocades NV, Delft, The Netherlands), and 100 U/ml streptomycin sulphate (Pharmachemie BV, Haarlem, The Netherlands).

The influenza-specific T cell clone K61-20 has been described elsewhere (22).

Immunofluorescence. Cells were sequentially incubated (in PBS containing 1% BSA and 0.02% sodium azide) with appropriate dilutions of the different antibodies and FITC-conjugated rabbit anti-mouse Ig (Dakopatts, Glostrop, Denmark) or FITC-conjugated swine anti-Rabbit Ig (Dakopatts) for 30 min at 0°C. For assaying the expression of CD44 variants on anti-CD3 (IgG2a subclass)-activated T cells, FITC-conjugated goat anti-mouse IgG1 subclass-specific Ig (Nordic Immunology, Tilburg, The Netherlands) was used. Fluorescence was measured by a FACScan® flow cytometer (Becton Dickinson & Co.).

Cell Proliferation. T cell proliferation was measured using a [^{3}H]thymidine incorporation assay. Anti-CD3-stimulated T cells were plated in 96-well flatbottomed tissue culture plates (Costar, Cambridge, MA) at 10^5 cells per well, and cultured for 1–7 d in RPMI 1640 with Hepes and 10% FCS. During the last 4 h of the assay, the culture was pulsed with 0.5 μCi [methyl-^{3}H]thymidine (87 Ci/mmol; Amersham International). Results are expressed as the arithmetic mean cpm of triplicate cultures.

RNA-PCR. 50–100 × 10^6 cells were harvested, pelleted, and dissolved in chaeotropic lysis buffer (3 M LiCl, 6 M Urea) and homogenized for 2.5 min at 0°C in a mixer (Omni International, Waterbury, CT) equipped with micro set-up at position 5.5. The homogenate was allowed to settle overnight at 0°C and subsequently the RNA was pelleted for 30 min at 25,000 rpm in a rotor (model SW60Ti; Beckman Instruments Inc., Fullerton, CA). The RNA pellet was dissolved in TES buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA) containing 1% SDS and 2 mg/ml proteinase K (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) and incubated for 30 min at 37°C. After phenol-chloroform extraction, the RNA was precipitated twice in ethanol and stored at −80°C in 70% ethanol until needed. RNA-PCR was performed on 1-μg aliquots of total RNA, after first strand cDNA synthesis using oligo dT primers, with ampliTaq DNA polymerase and CD44 primers according to the protocol provided with the RNA-PCR kit (Perkin-Elmer Corp., Norwalk, CT).

The primers were Hu-5′-EcoRV (exon v6) and Hu-3-EcoRV (exon v6), corresponding to positions 436–466 and 411–442 of the LCLC97 variant region as described by Hofmann et al. (9), and 5′ constant and 3′ constant, corresponding positions 513–540 and 900–922 of the standard CD44 sequence described by Stamenkovic et al. (14). Amplification was performed in 30 cycles (30 sec 95°C, 2 min 72°C) in a microwave-based DNA incubator (Ampliwave; Kreatech Biotechnology, Amsterdam, The Netherlands). PCR products were analyzed on a 1.5% ethidium bromide-containing agarose gel, subsequently transferred to nitrocellulose, and hybridized with a 5′ constant CD44-specific probe (9), or an exon v6-specific probe labeled with ^{32}P by nick translation.

Results

Characterization of Abs against the Variant Portion of the Human CD44 Glycoprotein. To obtain tools to study the expression and function of human variant CD44 glycoproteins, we raised polyclonal and monoclonal Abs to a fusion protein composed of variant exon-encoded sequences v3–v10 of human CD44 (Fig. 1, and Materials and Methods). As we have recently shown (12), the polyclonal rabbit antiserum obtained recognizes fusion proteins encoded by the variant exons v3–v10 found in immortalized keratinocytes, as well as fusion proteins encoded by only exons v3,4 or v6,7 in Western blots, and detects CD44 variants on several human epithelia.

For the generation of mAbs, we also immunized with the fusion protein encoded by exons v3–10 of human CD44 (Fig. 1, and Materials and Methods). To elucidate the exon specificity of the mAbs obtained, these mAbs were further tested against several bacterial fusion proteins in ELISA (not shown) and Western blot analysis (Fig. 2). mAb VFF6 reacted with fusion protein DII/III (encoded by exons v5,6), but not with fusion proteins DI (encoded by v3,4) and DIII (encoded by v6,7) (Fig. 2 *A*); mAbs VFF4 and VFF7 reacted with fusion

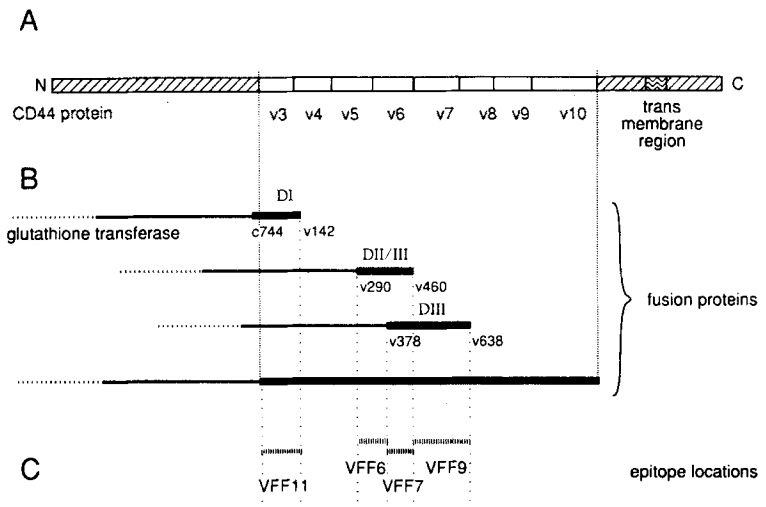

Figure 1. (*A*) Schematical representation of HPKII-type CD44v. (*Open boxes* v3–10) Exons that are expressed in immortalized keratinocytes (HPKII), but that are spliced out in (*hatched boxes*) standard CD44. (*B*) Schematical representation of bacterially expressed fusion proteins encoded by pGEX or pATH constructs (see Materials and Methods) CD44v HPKII (v3–10), DI, DII/III, and DIII. (*C*) Location of the epitopes that are recognized by the mAbs VFF6, VFF7 (and VFF4), VFF9, and VFF11.

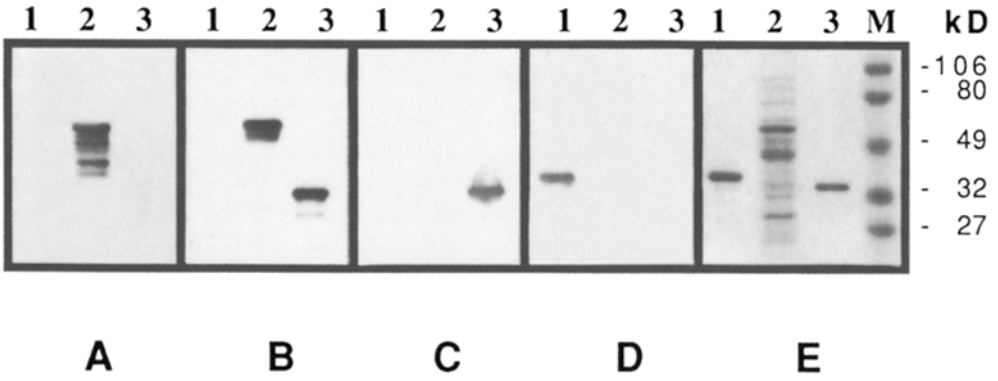

Figure 2. Western blot analysis of CD44v-encoded bacterial fusion proteins with different CD44v-specific mAbs. (*A–D*) Replica gels (10% polyacrylamide) carrying the following order of the fusion proteins: (lane *1*) DI, (lane *2*) DII/III, and (lane *3*) DIII. The figure shows staining of the fusion proteins with the mAbs (*A*) VFF6, (*B*) VFF7, (*C*) VFF9, and (*D*) VFF11. (*E*) Coomassie brilliant blue-stained SDS gel is shown. In lanes *1* and *3*, affinity-purified fusion proteins were loaded; lane *2* shows a crude bacterial lysate. (*M*) Size marker.

proteins DII/III and DIII, but not with DI (Fig. 2 *B*), and mAb VFF9 only recognized fusion protein DIII (Fig. 2 *C*). mAb VFF11 only showed reactivity with DI (Fig. 2 *D*). These data indicate that the five mAbs recognize four different epitopes on the variant portion of CD44, which are encoded by exons v3 or v4 (VFF11), v5 or v6 (VFF6), v6 (VFF4, VFF7), and v7 or v8 (VFF9), respectively. The position of these epitopes on the variant portion of CD44 is schematically depicted in Fig. 1 *C*.

Human Lymphohematopoietic Cells Express Low Levels of Variant CD44. In contrast to the strong expression of standard CD44-encoded epitopes (18), splice variants of CD44 were barely detectable on normal lymphohematopoietic cells and tissues. In tissue sections of tonsils, LNs, thymus, small and large intestine (containing mucosa-associated lymphoid tissue), lung, liver, and skin, we observed no unequivocal staining of lymphocytes and macrophages, including alveolar macrophages and Kupffer's cells, by immunoperoxidase with any of the antivariant CD44 Abs. It should be stressed that by the same technique, these Abs strongly stained epidermal keratinocytes of normal skin, as well as several other epithelia (12, and data not shown).

In FACS® analysis, polyclonal Abs against the complete variant region (v3–10), and mAbs VFF7 (and VFF4, not shown) against v6 weakly stained PBL, tonsillar lymphocytes, monocytes, and granulocytes, as well as the cell lines CEM, JVM-2, and U937 (Fig. 3). By contrast, except granulocytes, neither of these cells were stained with mAbs against epi-

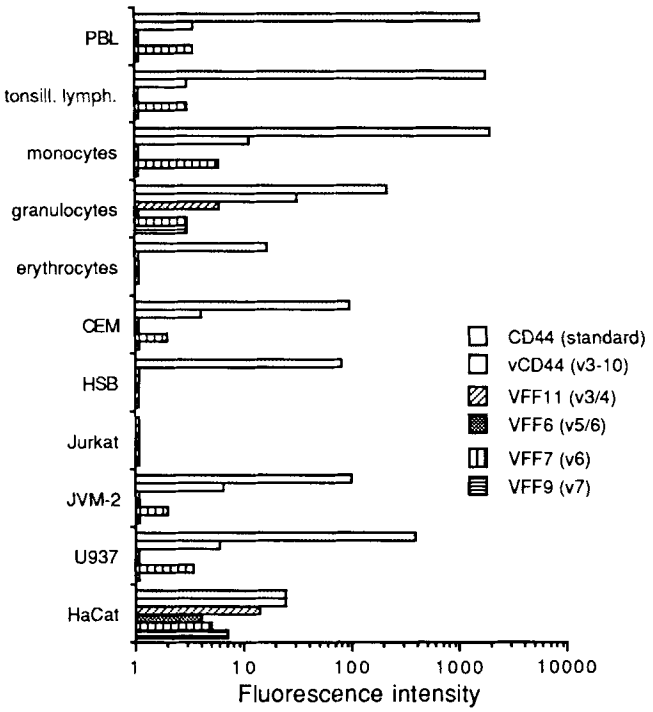

Figure 3. Human lymphohematopoietic cells and cell lines express low levels of variant CD44. Binding of different Abs was measured by FACS®. Results show mean fluorescence intensity. Negative controls (<5 intensity units in all cases) have been substracted. One single fluorescence peak was found in all cases.

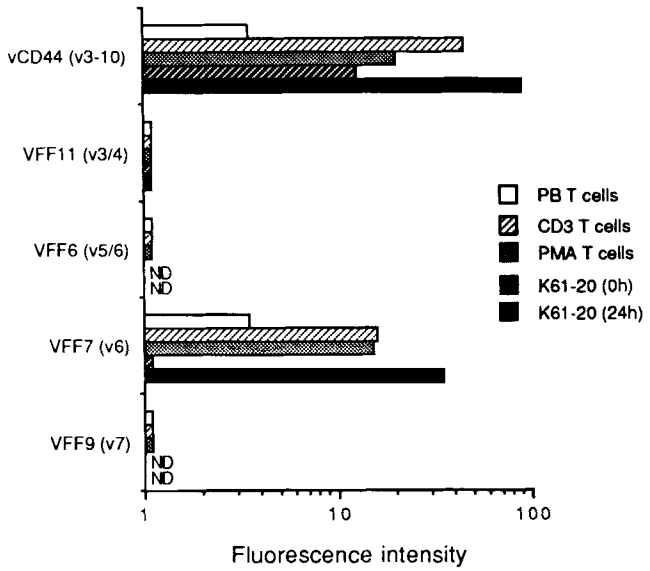

Figure 4. T cell activation by anti-CD3, PMA, or antigen leads to transient upregulation of CD44 variants containing the metastasis-associated domain coded by exon v6. (*PB T*) peripheral blood T lymphocytes (nonactivated); (*CD3 T* and *PMA T*) 24 h after activation with CD3 and PMA. (*K61-20*) Influenza-specific T cell clone.

topes encoded by v3/4 (VFF11), v5/6 (VFF6), or v7/8 (VFF9). The keratinocyte cell line HaCat, which was used as a positive control, stained with all of the antivariant CD44 Abs (Fig. 3). The data indicate that a very small proportion of CD44 as expressed on lymphocytes, monocytes, and granulocytes consists of splice variants that contain the metastasis-associated domain encoded by exon v6.

T Cell Activation Leads to Transient Upregulation of CD44 Variants Containing the Metastasis-associated Domain Encoded by v6. The fact that activation greatly influences both the adhesiveness and migratory properties of lymphocytes, prompted us to explore whether lymphocyte activation influences expression of CD44 splice variants. We observed that human peripheral blood T lymphocytes, upon activation with either anti-CD3 mAb and IL-2 or with the phorbol ester PMA, strongly upregulate the expression of variant CD44 glycoprotein(s) as detected by polyclonal Ab against CD44v (v3–10) (Fig. 4). Also, antigenic contact leads to strong upregulation of CD44v (v3–10) on the influenza-specific T cell clone K61–20 (Fig. 4). It is interesting that analysis of activated lymphocytes using the mAbs against various CD44 variant epitopes, gave positivity only with mAbs recognizing an epitope(s) encoded by metastasis-associated exon v6 (Fig. 4). Time kinetic studies showed that the upregulation of variant CD44 was transient peaking at 24–48 h and returning to background levels at day 3 (Fig. 5). Restimulation of the cells at day 6 led to reinduction of variant CD44 expression (data not shown). The enhanced expression of variant CD44 as found was accompanied by upregulation of IL-2R (CD25) (data not shown), and preceded proliferation as measured by [^{3}H]thymidine incorporation (Fig. 5). Hence, upon activation by phorbol ester, anti-CD3, or specific antigen, T lymphocytes show an early transient upregulation of CD44 variants containing the metastasis-associated domain encoded by v6.

PCR Analysis Demonstrates at Least Three v6-containing CD44 Splice Variants. To determine the level of regulation of variant CD44 expression and to elucidate the structure of the CD44 splice variants expressed in lymphocytes, RNA prepared from resting and activated T lymphocytes and several lymphoid cell lines was subjected to reverse transcription PCR amplification. In these studies, primers corresponding to sequences of the 5′ and the 3′ standard (constant) region and the v6 exon region were used. With the 5′ and 3′ constant primers, a major PCR product of ~450 bp was obtained from all cells except Jurkat (Fig. 6 *A*), which is also CD44 negative at the protein level (Fig. 3). The size of this product corresponds to that expected for the standard CD44 message. In addition, up to three minor bands of ~580, 680, and 860 bp were obtained from PBL, activated T lymphocytes, and CEM, but not from the CD44 variant negative cell line HSB (Fig. 6 *A*). Hybridization with a v6-specific probe shows that all of these three splice variants contain v6 (Fig. 6 *B*). These v6-containing PCR products were relatively most abundant at 24 and 48 h after T cell activation strongly, suggesting that expression of vCD44 is transcriptionally regulated (Fig. 6).

To gain insight into the structure of the CD44 variants, i.e., in which exons are flanking v6, we performed PCR analysis using v6-specific up- or downstream primers in combination with the 5′ and 3′ constant primers, respectively. Priming from v6 upstream yielded one PCR product of ~350 bp, indicating absence of v3–v5, whereas downstream priming yielded up to three major bands migrating at ~220, 550, and 770, and some minor bands, confirming the presence in lymphocytes of at least three splice variants with v6. These presumably contain either v6 alone (220), v6 with all other

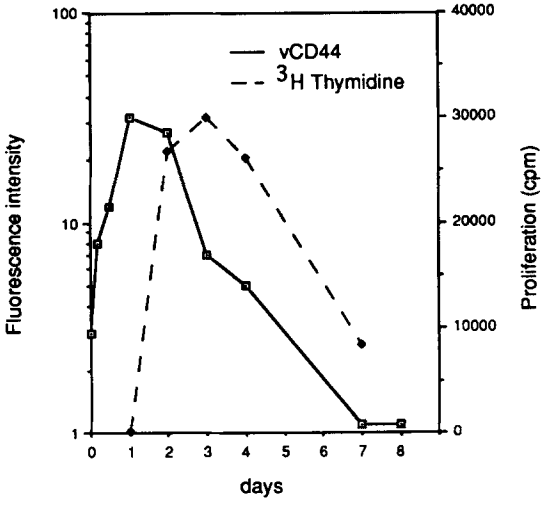

Figure 5. Time kinetics of variant CD44 upregulation (the epitopes seen by the polyclonal Ab to v3–10) in relation to [^{3}H]thymidine incorporation. Similar time kinetics were found for v6 expression.

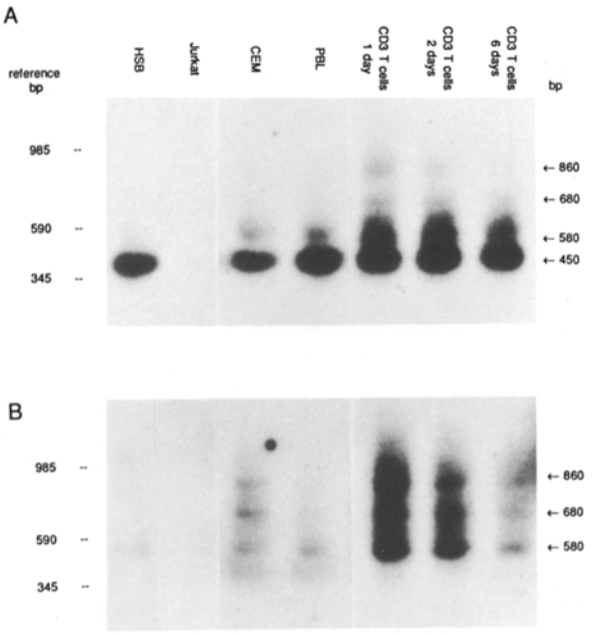

Figure 6. PCR amplification of cDNA with 5′ and 3′ CD44-specific primers. PCR products were transferred to nitrocellulose and hybridized with (*A*) CD44-specific 5′ probe, and (*B*) probe specific for CD44 variant exon v6. The probes were labeled with ^{32}P by nick translation.

Table 1. *Expression of CD44 Variants in Non-Hodgkin's Lymphomas*

Grade*	n	Antibody specificity (No. positives) v3–10	v3/4	v5/6	v6	v7/8
Low-grade						
Diffuse small cell	7	0	0	0	0	0
Follicular	5	0	0	0	0	0
Intermediate/High grade						
Diffuse large cell	14	7	0	0	6	0
Immunoblastic	5	3	0	0	2	0
Anaplastic (Ki-1)	5	4	0	0	2	0

* According to the WF (20).

downstream variant exons (v6–10), and v6 with an incomplete set of downstream variant exon (550).

Overexpression of CD44 Variants Containing the Metastasis Domain v6 in Aggressive Non-Hodgkin's Lymphomas. Prior studies have related expression of CD44 on NHLs to dissemination and to aggressive tumor behavior (3–5). At this time, a putative heterogeneity of CD44 molecules could not be assayed. This earlier finding, and the now recognized role of larger variants of CD44 in lymphogenic tumor spread (8), together with the present data showing that lymphocytes upregulate v6 upon activation, prompted us to pursue the possibility that NHLs express CD44 variants. Indeed, of a group of 36 NHLs selected for moderate to high expression of standard CD44 as detected by mAb NKI-P1, 14 tumors were found to express CD44 variant glycoproteins (Table 1). It is interesting that all of these vCD44$^+$ lymphomas belonged to the intermediate- and high-grade categories of the WF (20), which are characterized by aggressive clinical behavior and are therefore also termed "aggressive" lymphomas (23). In contrast, none of the low-grade NHLs expressed immunohistochemically detectable levels of CD44 variants. In view of the absence of immunohistochemically detectable levels of CD44 variants in normal lymphoid tissues, these findings indicate a marked overexpression in the positive tumors. The level of variant expression in those tumors was variable ranging from weak to strong. 10 of 14 CD44 variant positive tumors showed expression of v6-encoded epitopes (Table 1 and Fig. 7). Three tumors that stained weakly with the polyclonal antiserum did not react with any of the mAbs. Hence, 10 of 24 CD44$^+$ aggressive lymphomas overexpress CD44 variants containing the metastasis associated domain v6.

Discussion

Lymphohematopoietic cells have been known to express the standard, so-called hematopoietic (14) form of CD44, that

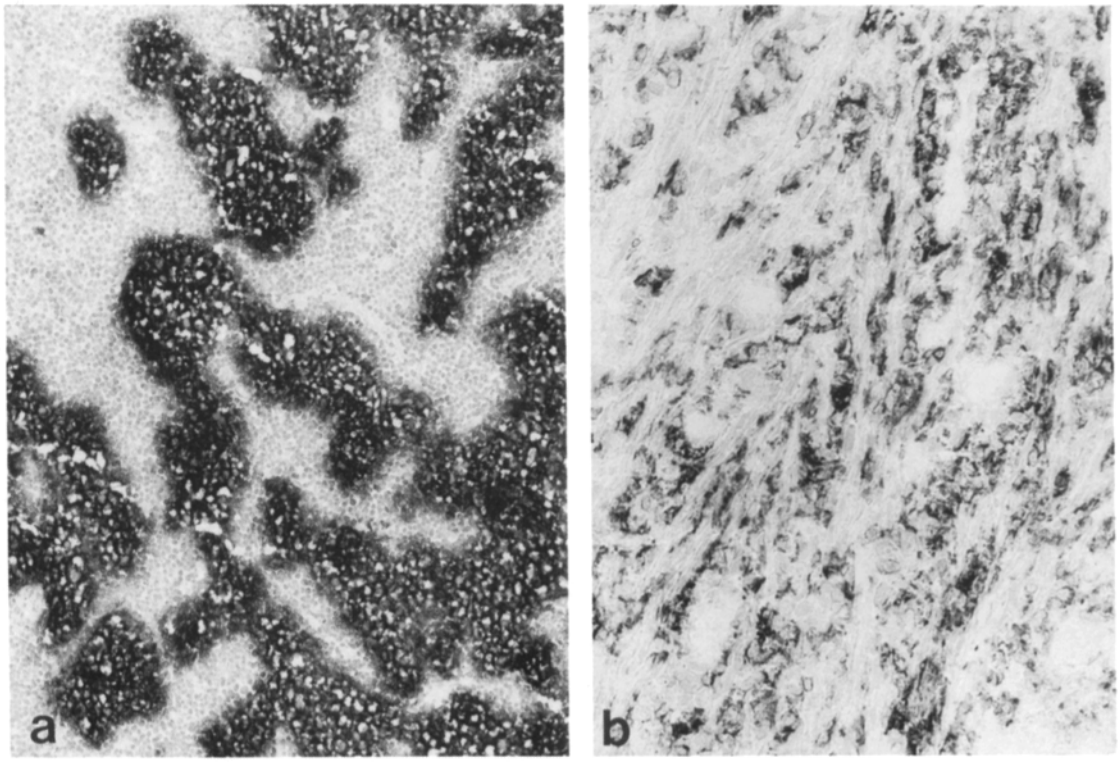

Figure 7. Expression of the metastasis-associated variant of CD44 on aggressive human NHLs. (*A*) High-grade (anaplastic) B cell lymphoma stained with mAb VFF7 against v6. Note that preexisting nonneoplastic lymphocytes in this LN show no detectable staining. (*B*) High-grade T cell lymphoma infiltrating into the muscularis propria of the bowel wall, stained with mAb VFF7 (V6). (×125).

has been implicated in facilitating lymphocyte migration from the blood into lymphoid tissues (24, 25). After antigenic stimulation in vivo, rat lymphocytes have recently been shown to synthesize one specific variant of CD44 carrying the v6 exon (11). By using Abs raised against the variant portion of the human CD44 protein, we now demonstrate that human leukocytes express low levels of CD44 splice variants and that human T lymphocytes, can be stimulated in vitro to transiently upregulate expression of variant epitopes. These variants, which contain sequences that in the rat confer metastatic behavior to carcinoma and sarcoma cells (8), are overexpressed in about half of the studied aggressive NHLs.

Whereas biochemical studies indicated the presence of heterogenous sets of CD44 glycoproteins on many cell types including lymphohematopoietic cells (18, 26, 27), and several CD44 RNA splice variants have recently been identified (8, 9, 12, 14, 28–31) our present study documents regulated differential expression at the protein level of variant domains in lymphohematopoietic cells. These variants are expressed at very low levels on normal resting lymphocytes, granulocytes and monocytes, and on several lymphohematopoietic cell lines (Fig. 3), in contrast to the abundant expression of standard CD44 on those cells (Fig. 3). However, T cell activation either by anti-CD3 mAb, phorbol ester PMA, or specific antigen, leads to a transient upregulation of variant CD44 glycoproteins (Fig. 4), which peaks at 24 h and precedes blast transformation and S phase (Fig. 5). A similar upregulation of CD44 splice variants can be found upon activation of B lymphocytes (Griffioen, A., manuscript in preparation). These data are compatible with our previous observations in rats that showed CD44 variant expression upon in vivo stimulation by antigen (11).

It is interesting that among the CD44 variant glycoproteins expressed by activated T lymphocytes, those containing the metastasis-associated exon v6, predominate (Figs. 3 and 4). PCR analysis of the variant transcripts revealed regulation of variant expression at the transcriptional level and demonstrated the existence of at least three transcripts containing v6, consisting of either v6 alone or v6 in combination with several or all downstream exons v7–10 (Fig. 6). In the rat, only one $v6^+$ PCR product has been detected in lymphocytes sorted for the epitope (11). This discrepancy might reflect interspecies differences or might be caused by different modes of stimulation. Of the v6-containing transcripts, only the longest transcript has been described previously (9), but in this study, no lymphoid cells were included. It remains to be determined whether the other variants are specific for lymphocytes. In the rat, variants containing v6 are part of the process of metastasis formation (8). Hence, at an early stage of activation, T lymphocytes acquire molecules which in another setting can promote tumor metastasis.

The insertion of exon-encoded sequences into the membrane-proximal region of CD44 on activated T lymphocytes may serve a number of different functions. For example, spliced exons could modulate the ligand-binding properties of CD44. Indeed, it has been shown by Aruffo et al. (32) that the presence of a 135-amino acid insert within the membrane-proximal region, as found in the so-called epithelial variant of CD44 (corresponding to v8–10 in Fig. 1) dramatically reduces hyaluronic acid binding, and lymphocyte adhesion to cultured high endothelial venules (25). Binding of CD44 to its other putative ECM ligands collagen (33) and fibronectin (34) might be modulated in a similar way. Alternatively, the introduction of new sequences into the CD44 molecule might create entirely new binding specificities. In this way, CD44 might contribute to the structural basis of tissue-specific homing.

We observed that a significant proportion of aggressive NHLs overexpress CD44 variants that carry the metastasis-associated exon v6 (Table 1, Fig. 7). Unlike the low-grade lymphomas, which have preserved more functions of their nonneoplastic counterparts such as homing pattern, and responsiveness to immunoregulation, and usually follow an indolent clinical course, many lymphomas of the intermediate and high-grade categories of the WF (20) have an aggressive natural history. They are autonomous neoplasms showing destructive growth and behaving in a fashion similar to that of other highly malignant nonlymphoid tumors (20, 23). Whereas standard CD44 is expressed on most lymphocytes and lymphomas and may have a role in homing to lymphoid tissues, activated lymphocytes and aggressive lymphomas produce v6 variants. They share infiltrative, adhesive, and migratory properties. It is likely that v6 has a role in this aggressive behavior by either recruiting new molecular partners, by changing CD44 properties, and/or by pleiotropic actions (growth advantage and induction of an array of relevant functions).

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