Evaluation of Soluble CD44v6 as a Potential Serum Marker for Head and Neck Squamous Cell Carcinoma


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ABSTRACT

In recent years, the measurement of soluble CD44 levels in the circulation of patients with malignant diseases has been introduced as a new and simple diagnostic tool for the detection of human cancer. The high CD44v6 expression in head and neck squamous cell carcinoma (HNSCC) would enable the use of soluble CD44v6 proteins present in the circulation of HNSCC patients as a marker of disease. In the present study, we determined CD44v6 plasma levels using a domain-specific ELISA in healthy volunteers, non-cancer patients, and HNSCC patients before and after surgical removal of the tumor. A difference between the CD44v6 plasma levels of HNSCC patients and controls could not be observed. Moreover, surgical removal of the tumor did not result in a reduction of the CD44v6 plasma level in the HNSCC patients. In addition, the spectrum of soluble v6-containing CD44 proteins present in the plasma of HNSCC patients and controls was determined by immunoprecipitation experiments, but again, tumor-related isoforms could not be distinguished in patient samples.

Additional experiments to unravel the biological source of these circulating proteins indicated surprisingly that the v6-containing proteins present in the circulation of healthy individuals are only released in part, if at all, by activated lymphocytes or other nucleated blood cells. Most circulating CD44v6 proteins seem to be derived from the normal epithelial cell compartments, including breast cells, colon cells, and squamous cells. Taken together, these data do not support the use of soluble CD44v6 as a tumor marker in HNSCC or any other tumor type that has developed from tissues producing soluble isoforms.

INTRODUCTION

CD44 is a cell surface glycoprotein expressed by a large variety of tissues. A large family of CD44 isoforms exists, encoded by tissue-specific alternatively spliced transcripts. The CD44 isoforms differ in the size of their extracellular or intracellular domain and their tissue distribution. Multiple functions have been ascribed to CD44, but they appear to be restricted to subsets of the various isoforms. First, CD44 acts as a receptor for the extracellular matrix components hyaluronate, laminin (3), fibronectin, and type I and VI collagen (4). In addition, CD44 is thought to play a role in several processes critical in normal immune system development and functioning, including lymphocyte homing (5, 6), hematopoiesis (7), and leukocyte activation (8–10). Finally, CD44 molecules containing the v3 domain are able to bind growth factors and might play a role in the control of cell proliferation (11).

In addition to their expression on the cell membrane, CD44 proteins are also released from cells, and soluble CD44 proteins are detectable in the normal human circulation (12–15). The basic level of soluble CD44 in the circulation is thought to have its origin in lymphocytes (16–18). The observation that several human tumors show an overexpression of certain CD44 isoforms and the presence of soluble CD44 isoforms in the circulation make CD44 a potential serum marker for tumor detection. Elevated CD44 serum levels have indeed been observed for several tumor types using ELISAs with domain-specific antibodies directed against the standard form of CD44: (a) CD44s (19–21); (b) CD44v5 (22, 23); or (c) CD44v6 (20, 22–24). Compared to other tumor types, the CD44v6 domain is highly expressed by HNSCC,2 and squamous cell carcinomas of the lung, esophagus, cervix, and skin (25). MAb U36, which recognizes the v6 domain of CD44, stains more than 50% of the cells intensely in 188 of 196 (96%) HNSCC biopsies (26). Therefore, this domain seems to be a promising candidate serum marker for squamous cancers like HNSCC.

To evaluate whether plasma levels of soluble CD44v6 have diagnostic significance for HNSCC, a longitudinal pilot study was performed with 13 HNSCC patients. Plasma levels of soluble CD44v6 were measured with a v6-specific ELISA assay before and after surgical removal of the tumor and compared with the plasma levels of non-cancer patients and healthy volunteers. To distinguish the various circulating isoforms and investigate the presence of specific tumor-derived soluble forms, the spectrum of circulating CD44v6 proteins was assayed.

Received 5/3/99; revised 8/9/99; accepted 8/9/99.

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2 The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; MAb, monoclonal antibody; PHA, phytohemagglutinin; RT-PCR, reverse transcription-PCR.
by immunoprecipitation experiments on the plasma of controls and HNSCC patients. Finally, the origin of the circulating CD44v6 isoforms was assessed by immunoprecipitation experiments with various domain-specific anti-CD44 antibodies and particular model experiments.

**MATERIALS AND METHODS**

**Plasma of HNSCC Patients, Non-Cancer Patients, and Healthy Volunteers.** Blood was obtained from 13 HNSCC patients (Table 1) and 10 non-cancer patients scheduled for surgery as well as from 8 healthy volunteers using heparin vacutubes (Becton Dickinson Vacutainer Systems Europe, Meylan, France). All patient samples were taken just before surgery from an arterial line. Additional blood samples were taken from the HNSCC patients directly after surgical removal of the tumor and subsequently spun to remove the cells (30 min, 80°C using intensifying screens). The study was approved by the Medical Ethical Committee of the University Hospital Vrije Universiteit.

**CD44v6-specific ELISA.** A commercially available sandwich-type ELISA developed by Bender MedSystems (Vienna, Austria) was used for the quantitative measurement of soluble CD44v6 in plasma samples. In this test, a MAb specific for an epitope of the CD44 standard molecule is used as the capturing antibody adsorbed onto microtiter plates. The anti-v6 antibody VFF18 coupled to horseradish peroxidase serves as tracer antibody with the peroxidase/tetramethyl-benzidine substrate system for readout. The ELISA test was performed according to the manufacturer’s instructions.

In short, 100 μl of the plasma samples (diluted 1:20 in sample diluent), standard solutions, and negative controls (sample diluent) were added to the wells, together with 50 μl of the tracer antibody. Subsequently, plates were incubated for 3 h at room temperature on a rotator platform, allowing the tracer and capture antibodies to bind simultaneously with the CD44 present in the standards and plasma samples. Unbound CD44 and tracer antibody were removed by three washing steps, and 100 μl of substrate solution were then added for color development. After 15 min, the reaction was terminated by the addition of 100 μl of 2 M H2SO4, and color intensity was measured at 450 nm (ELISA reader Anithos ht-II; Anithos Labtec Instruments, Salzburg, Austria). Since the plasma samples were diluted twice with RPMI 1640 (Bio-Whittaker), and RPMI 1640 alone did not give any signal in the assay, the measured CD44v6 levels were multiplied by 2 to calculate the actual plasma concentration.

MAbs. Anti-CD44 MAb SFF304 (anti-CD44s), VFF327v3 (anti-CD44v3), VFF8 (anti-CD44v5), VFF18 (anti-CD44v6), VFF9 (anti-CD44v7), VFF17 (anti-CD44v7,8), FW3 11.24 (anti-CD44v9), and VFF14 (anti-CD44v10) were obtained from Bender MedSystems.

**Immunoprecipitation of CD44 Antigen.** CD44 antigen was precipitated with goat antimouse IgG-agarose beads (Sigma, Bornem, Belgium) saturated with anti-CD44 MAbs. Before antibody-coupling, antimouse IgG agarose was washed twice with PBS and blocked with 1% BSA in PBS (1% BSA/PBS) by overnight incubation at 4°C. For MAbs VFF18 (anti-CD44v6) and SFF304 (anti-CD44s), 50 μl of antimouse IgG agarose were used, and for MAbs VFF327v3 (anti-CD44v3), VFF8 (anti-CD44v5), and FW3 11.24 (anti-CD44v9), 100 μl of antimouse IgG agarose were used. After washing once with PBS, the agarose beads were incubated for 8 h at 4°C with 5 μg (VFF18, SFF304) or 50 μg (VFF327v3, VFF8, FW3 11.24) tracer MAb diluted in a final volume of 500 μl of 0.01% BSA/PBS. Uncoupled antibody was removed by washing the saturated agarose beads three times with PBS. Subsequently, the preformed agarose-MAB complexes were incubated overnight at 4°C with the sample (50 μl of human plasma adjusted to a final volume of 500 μl with PBS, 500 μl of murine plasma, or 1 ml of lymphocyte culture medium). The next morning, agarose beads were washed six times with PBS, and, finally, 20 μl of sample buffer [50 mM Tris (pH 6.8), 4% SDS, 12% glycerol, 0.01% bromphenol blue, and 5% β-mercaptoethanol] were added. Samples were boiled for 5 min before loading on the gel. Immunoprecipitations with MAbs VFF17 (anti-CD44v7,8), VFF9 (anti-CD44v7), and VFF14 (anti-CD44v10) were not successful, probably due to the relatively low affinity of these antibodies.

**Western Blot Analysis.** Immunoprecipitated proteins were separated by SDS-PAGE on a 5% gel and transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The free binding sites were blocked by incubating the blot overnight with 1% BSA/PBS (27). The filter was subsequently incubated for 1 h with 0.75 μCi/ml 125I-labeled MAb VFF18 (3.5 μCi/μg MAb) in 1% BSA/PBS (25). Unbound MAb was removed by washing three times for 15 min in PBS with 0.05% Tween 20, and bands were visualized by autoradiography for 48–64 h at ~80°C using intensifying screens. **Analysis of CD44 Expression on in Vitro Stimulated Lymphocytes.** Blood (30 ml) from a healthy control was collected in heparin vacutubes (Becton Dickinson Vacutainer Systems Europe, Meylan, France). All patient samples were taken just before surgery from an arterial line. Additional blood samples were taken from the HNSCC patients directly after surgical removal of the tumor and subsequently spun to remove the cells (30 min, 80°C using intensifying screens).
Soluble CD44v6 in HNSCC

Lymphocytes were isolated by density separation using lymphoprep (Nycomed, Oslo, Norway). In short, blood was diluted twice with RPMI 1640 (Bio-Whittaker). The diluted blood was then carefully applied onto 30 ml lymphoprep (Nycomed), resulting in two separate phases. After centrifugation (20 min, 900 × g), lymphocytes (present at the interface) were collected with a sterile glass pipette and washed twice with RPMI 1640 (Bio-Whittaker). In total 1.1 × 10^7 lymphocytes were isolated. Half of the cells were resuspended in RPMI 1640 (Bio-Whittaker) supplemented with 10% FCS (Hyclone, Logan, UT), 1% penicillin, 1% streptomycin, and 2% PHA (Gibco Life Technologies B.V., Breda, the Netherlands) to a final concentration of 2.75 × 10^6 cells/ml, and the other half of the cells were resuspended to the same concentration in RPMI 1640 (Bio-Whittaker) supplemented with 10% FCS (Hyclone), 1% penicillin, and 1% streptomycin. Cells were plated in a 24-well plate (0.5 ml/well) and cultured under 5% CO2 at 37°C. After 4 days, the lymphocytes were collected by centrifugation (10 min, 300 × g) and washed twice with PBS. Twenty μl of ice-cold lysis buffer [20 mM Tris-HCl (pH 7.5), 140 mM NaCl, 4 mM EDTA, and 1% NP40] with protease inhibitors (10 mM phenylmethylsulfonyl fluoride, 1 mM iodo acetamid, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 20 μg/ml trypsin inhibitor) were added per 1 × 10^7 lymphocytes. After a 30-min incubation on ice, cell debris was removed by centrifugation (30 min, 4°C, 25,000 × g). Cell lysates were stored at −20°C until use. For Western blotting, 15 μl of cell lysate were loaded on the gel. The culture medium of the lymphocytes was spun in an Eppendorf centrifuge (1 h, 4°C, 25,000 × g) to remove the cell debris and stored at −20°C until immunoprecipitation. For immunoprecipitation of soluble CD44 isoforms, 1 ml of conditioned culture medium was used.

**Isolation of RNA from Nucleated Cells Present in Blood and Bone Marrow.** Blood (7 ml) and bone marrow (1–2 ml) were collected in heparin vacutubes (Becton Dickinson Vacutainer Systems Europe). Blood and bone marrow were diluted twice with RPMI 1640 (Bio-Whittaker) and spun (10 min, 220 × g, 4°C). The supernatant was removed, and the remaining cells were resuspended in 10 (blood) or 5 ml (bone marrow) of shock buffer [0.16 M NH4Cl, 10 mM KHCO3, and 0.1 mM Na2EDTA (pH 7.4)] to lyse the erythrocytes. After a 10–15 min incubation at 4°C, the nucleated cells were spun down (5 min, 220 × g, 4°C) and washed twice with RPMI 1640 (Bio-Whittaker). Subsequently, RNA was isolated using 1 ml of RNazol (Campro Scientific B.V., Veenendaal, the Netherlands) according to the manufacturer’s instructions.

**Isolation of RNA from v6-positive Cells of Normal Oral Mucosa and Normal Colon Cryosections.** CD44v6-positive cells were microdissected from cryosections (10 μm) of a normal oral mucosa and colon sample. To allow selection of the v6-positive cells, parallel cryosections were immunohistochemically stained with anti-v6 MAb U36 and VFF18 using a biotin-streptavidin immunoperoxidase staining technique, as described previously (26). For orientation, the sections that were used for microdissection were stained for 15 s with a solution containing 1% toluidine blue and 0.2% methylene blue. After rinsing with Millipore filtered water, microdissection was carried out immediately using a Zeiss Stemi SV 11 stereomicroscope. For each sample, two to five areas of tumor or normal tissue were dissected, depending on the size of the regions. Microdissected tissue was stored on ice until RNA was isolated.

The RNA was extracted with RNazol (Campro Scientific B.V.) essentially according to the manufacturer’s instructions. The procedure was scaled down to a volume of 100 μl of RNazol, and glycogen (5 μg) was added as a carrier for the subsequent precipitation. The RNA pellets of the microdissected tissues were dissolved in 25 μl of water.

**RT-PCR and Southern Blot Hybridization.** First-strand cDNA synthesis was carried out using 0.5 μg of total RNA isolated from nucleated cells of blood and bone marrow or 5 μl of total RNA isolated from microdissected samples as a template. The RNA samples were heated for 3 min at 70°C and immediately put on ice. The RNA was reverse transcribed to cDNA by avian myeloblastosis virus reverse transcriptase (Promega Corp., Leiden, the Netherlands), using an antisense primer complementary to a sequence in exon 17 of the CD44 gene (exon 17 antisense primer, 5’-CAAGACAAAAGCGCAAGGGAAT-3’). The reverse transcription reaction was performed in a volume of 20 μl and contained 1 mM deoxynucleotide triphosphates, 1 mM DTT, 50 mM Tris-HCl (pH 8.3), 60 mM KCl, 3 mM MgCl2, 1.25 μM antisense primer, 2 units of RNasin (Promega Corp.) and 0.75 unit of avian myeloblastosis virus reverse transcriptase (Promega Corp.). The reactions were incubated for 2 h at 42°C.

The obtained cDNA products were used as a template for PCR amplification using the antisense primer indicated above and a sense primer identical to a sequence in exon 5 (exon 5 sense primer, 5’-TGTCCAGAAAGGAGAATACAGAACG-3’). The PCR was performed in a total volume of 50 μl, using 5 μl of the cDNA product. The final reagent concentrations for RT-PCR were 200 μM deoxynucleotide triphosphates, 0.625 μM antisense primer, 0.5 μM sense primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM MgCl2. Water was added to the negative control instead of RNA template. Strict precautions were taken to prevent carryover contamination. PCR mix pipetting, RNA isolation/cDNA synthesis, and PCR amplification were carried out in three separate laboratories. The PCR conditions used were as described by Hudson et al. (28) with a hot start of 5 min at 95°C and cooling to 72°C. Subsequently, 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer, Gouda, the Netherlands) was added. This was followed by 3 min at 57°C; 3 min at 72°C; 30 cycles of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C; and a final incubation of 4 min at 72°C. PCR amplification was performed on a Hybaid OmniGene thermal cycler (Biozym, Landgraaf, the Netherlands) with paraffin oil to prevent evaporation. Ten μl of the reaction mixtures were loaded on a 1% agarose gel and transferred to a charged nylon membrane (Qiagen Westburg B.V., Leusden, the Netherlands) using a transfer buffer containing 0.4 M sodium hydroxide and 0.6 M sodium chloride according to the protocol of Sambrook et al. (27). The filters were baked for 2 h at 80°C, prehybridized for 1 h at 65°C in hybridization mix containing 0.5 mM NaHPO4 (pH 7.2), 7% SDS, and 1 mM EDTA, and subsequently hybridized overnight at 65°C after the addition of 32P-labeled v6 exon as a probe. After two washes with a 2× saline sodium citrate buffer containing 0.1% SDS (2× SSC/0.1% SDS) and two washes with 0.2× SSC/0.1% SDS at 65°C, the blot was exposed to x-ray film.
to Kodak X-AR 5 film and autoradiographed overnight at 
−80°C using intensifying screens.

**Plasma of Xenograft-bearing Nude Mice.** The tumor xenograft cell line HNX-OE was established from a metastasis of a squamous tumor of the oral cavity. Fresh biopsies of the tumor were implanted s.c. in the lateral thoracic regions on both sides of athymic nude mice. The xenograft cell line was maintained by serial s.c. transplantation. Blood samples from xenograft-bearing nude mice (tumor size, about 1000 mm³) and control nude mice were taken in heparin vacutubes (Becton Dickinson Vacutainer Systems Europe) and spun (30 min, 1400 × g, 4°C). Plasma samples were stored at −80°C until further analysis. For immunoprecipitation, 500 μl of murine plasma were used.

**RESULTS**

**Level and Spectrum of Soluble CD44v6 Variants in Plasma of HNSCC Patients and Controls.** In a pilot study, the prognostic value of soluble CD44v6 levels in the blood of HNSCC patients was determined. The CD44v6 protein levels were measured in the plasma of 13 HNSCC patients, 10 non-cancer patients, and 8 healthy volunteers by an isoform-specific ELISA (Fig. 1). Longitudinal samples from 13 HNSCC patients taken just before, directly after, and 6 weeks after surgical removal of the tumor were analyzed. As indicated in Fig. 1, the CD44v6 plasma levels directly after surgical removal of the tumor (P2; 103 ± 46 ng/ml) were lower than those just before surgery (P1; 129 ± 37 ng/ml), a difference that appeared to be significant (paired t test, P = 0.0066). However, 6 weeks after surgery, the CD44v6 plasma levels had increased (P3; 167 ± 53 ng/ml). This increase appeared to be significant when compared with the initial plasma levels (paired t test, P = 0.0066) or with the plasma levels of the samples taken directly after surgery (paired t test, P = 0.000045). From this initial study, we could already conclude that the CD44v6 level in plasma is not related to the presence of tumor because removal of the malignant tissue resulted in an increase in CD44v6 levels. The observed changes are most likely caused by blood dilution as a result of fluid infusion during the operation. This conclusion is further substantiated by the observation that plasma levels of HNSCC patients just before surgery (P1; 129 ± 37 ng/ml) were not significantly different (unpaired t test, P = 0.36) from those of non-cancer patients that had received comparable volumes of fluid by infusion (CP; 109 ± 62 ng/ml). Furthermore, a significant difference in CD44v6 plasma levels (unpaired t test; P = 0.13) could not be determined between blood samples taken from HNSCC patients 6 weeks after surgery (P3; 167 ± 53 ng/ml) and samples taken from 8 healthy volunteers (HV; 134 ± 33 ng/ml). Together, these results clearly demonstrate that the CD44v6 plasma levels of HNSCC patients are not related to the presence or absence of tumor, but merely to the physiological conditions during sampling.

Although significant quantitative differences could not be observed between the CD44v6 plasma levels of HNSCC patients and controls, there could still be a difference in the spectrum of v6-containing CD44 isoforms present in the circulation. It has been suggested previously that soluble CD44v6 in the circulation of healthy controls originates from activated lymphocytes (18). We have previously shown that the v6 splice variants (in fact, all splice variants) expressed by HNSCC and normal oral mucosa are identical with respect to both the level of expression and the spectrum. However, these splice variants are distinct from the v6 variants expressed by activated lymphocytes, and additional squamous cell-specific variants released by HNSCC tumors might be detectable in the plasma of HNSCC patients. To compare the spectrum of v6-containing isoforms present in the plasma of non-cancer patients and HNSCC patients, immunoprecipitation experiments were performed (Fig. 2). CD44 isoforms were precipitated with anti-CD44v6 MAb VFF18. Subsequently, a Western blot of the precipitated CD44v6 proteins was incubated with 125I-labeled anti-CD44v6 (VFF18) to detect the v6-containing CD44 proteins. Protein bands of about M, 190,000, M, 155,000, M, 140,000, M, 125,000, and M, 95,000 could be detected in the samples of both non-cancer patients and HNSCC patients (Fig. 2, A and B, respectively). The protein bands of M, 190,000 and M, 155,000 were clearly visible for the samples of all non-cancer patients and all HNSCC patients. The protein bands of M, 140,000, M, 125,000, and M, 95,000 differed in intensity between individual samples of both HNSCC patients and non-cancer patients. From these experiments, we had to conclude that specific soluble isoforms produced by the tumor could not be observed in HNSCC patients. This could be due to the fact that HNSCC cells do not produce soluble forms. To study the release of CD44v6 from HNSCC cells in vivo, we used a model of HNSCC xenograft-bearing nude mice. The results of immunoprecipitation experiments on the plasma of HNSCC xenograft-bearing nude mice and control mice are depicted in Fig. 3. Proteins were precipitated with anti-CD44v6, and, subsequently, a Western blot of the precipitated CD44v6 proteins was incubated with 125I-labeled anti-CD44v6 for detection of the v6-containing CD44 proteins. In the plasma of control mice, no v6-containing proteins were detectable, which is in agreement with the species-specific recognition of CD44v6 by MAb VFF18. Therefore, the soluble v6-containing proteins that were detected in the plasma of the xenograft-bearing mice originated...
from the human HNSCC xenografts. The protein pattern appeared to be similar, at least in part, to the pattern observed for normal human plasma (data not shown). These data indicate that malignant squamous cells are indeed capable of producing soluble forms, at least in this model system.

**Origin of Soluble CD44v6 Proteins Present in Normal Human Plasma.** Our results show that soluble CD44v6 proteins cannot be used for tumor detection in HNSCC patients, although they appear to be produced, because non-cancer control patients have similar CD44v6 plasma levels consisting of identical variants. This observation was unexpected because the soluble proteins in normal human plasma were suggested to be derived from activated lymphocytes, thus additional squamous-specific variants in the plasma of HNSCC patients were supposed to be present. We therefore characterized the soluble forms identified in human plasma in more detail by mapping the assortment of domains. Immunoprecipitation was performed with anti-CD44s, anti-CD44v3, anti-CD44v5, anti-CD44v6, and anti-CD44v9. Subsequently, a Western blot of these immunoprecipitates was incubated with $^{125}$I-labeled anti-CD44v6 to detect the proteins containing the v6 domain. Similar results were found for the plasma of HNSCC patients and non-cancer patients (data not shown), indicating that the soluble CD44v6 variants are indeed identical. A typical example is shown in Fig. 3. The protein bands of $M_r$ 190,000 and $M_r$ 140,000 were present after precipitation with antibodies against the standard domain and the variable domains v3, v5, v6, and v9 of CD44. The $M_r$ 155,000, 125,000, and 95,000 protein bands were only precipitated with anti-CD44s, anti-CD44v6, and anti-CD44v9 antibodies, and not with anti-CD44v3 or anti-CD44v5.

To investigate whether the CD44 protein release from human lymphocytes can indeed explain the presence of all soluble CD44v6 proteins in human plasma, in vitro cultured lymphocytes were studied. Human lymphocytes were isolated from the blood of a healthy control person and cultured for 4 days in medium with or without PHA, which activates the lymphocytes. Cell lysates and conditioned culture medium were analyzed for the presence of v6-containing CD44 proteins. Al-

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**Fig. 2** Comparison of soluble CD44v6 proteins in the plasma of non-cancer patients and HNSCC patients. CD44v6 proteins present in the plasma of seven non-cancer patients (A) and six HNSCC patients (B) were immunoprecipitated with anti-CD44v6 MAb VFF18. Detection of immunoprecipitated CD44 proteins was performed by incubation of the Western blot with $^{125}$I-labeled VFF18.

**Fig. 3** Release of CD44v6 proteins from HNSCC xenografts. CD44v6 proteins present in the plasma of a HNSCC xenograft-bearing nude mouse (Lane +SCC) and a control nude mouse (Lane 0) were precipitated with anti-v6 MAb VFF18. Detection of the precipitated CD44 proteins was performed by incubation of the Western blot with $^{125}$I-labeled VFF18. For immunoprecipitation, 500 μl of murine plasma were used.

**Fig. 4** Characterization of the v6-containing soluble proteins in the plasma of a HNSCC patient (patient 4, Fig. 2B). Immunoprecipitation was performed with a panel of anti-CD44 MAbs recognizing different variant domains (anti-CD44s, anti-CD44v3, anti-CD44v5, anti-CD44v6, and anti-CD44v9). Detection of immunoprecipitated CD44 proteins was performed by incubation of the Western blot with $^{125}$I-labeled VFF18.
though CD44v6 proteins were clearly up-regulated in lymphocytes that had been activated by PHA, soluble CD44v6 proteins could hardly be detected in conditioned culture medium of lymphocytes cultured in either the absence or presence of PHA (data not shown). These data indicate that the release of CD44 from activated lymphocytes in vitro is low. However, the possibility that release of CD44 isoforms is different in vivo cannot be excluded.

To identify the main v6-containing CD44 proteins expressed by activated lymphocytes and other nucleated blood cells, an RT-PCR reaction was performed on RNA isolated from the nucleated cells present in the blood and bone marrow of two non-cancer patients. The assortment of exons in the observed splice variants should explain the domains in the immunoprecipitated isoforms. If not, then we have to conclude that the soluble CD44v6 isoforms are derived from other cells and tissues. Fig. 5A shows the RT-PCR results when using a sense primer in exon 5 and an antisense primer in exon 17, amplifying all variant exons of CD44. The amplimers were run on an agarose gel, blotted, and hybridized with exon v6 as probe. In blood and bone marrow samples, two main PCR products were hybridizing: (a) a fragment of 500 bp; and (b) a fragment of 375 bp. Subcloning and sequencing revealed that the 500-bp PCR product resulted from amplification of a CD44 splice variant that only contained variant exon v6 (calculated size of the PCR product, 508 bp). Subcloning and sequencing of the 375-bp PCR product showed that this fragment originates from CD44s (calculated size of the PCR product, 379 bp). Therefore, the positive 375-bp signal on the blot appears to be derived from unspecified cross-hybridization, which was apparent on the clones as well. This apparent cross-hybridization could not be explained because the sequence similarity between CD44s and the probe is only 60% for both the sense and antisense strand. Only the relatively large amount of the 375-bp CD44s PCR product, as visualized by ethidium bromide staining of the agarose gel, could explain this phenomenon. Note that in the colon, CD44s cross-hybridization is weak, and in HNSCC, it is absent (Fig. 5, B and C). This isoform is hardly expressed in the latter tissue. In addition to the 375- and 500-bp fragments, some larger PCR products were faintly detectable in the blood and bone marrow samples after a long exposure of the blot.

The observation that the soluble CD44 variants that are the most abundant in human plasma also contain the domains v3, v5, and v9 indicates that activated lymphocytes or other types of nucleated blood cells are not the only source of soluble v6-containing proteins. It appears that other cell types are also involved. Because the v6 domain of CD44 is predominantly expressed by epithelia, such as the skin, lungs, oral mucosa, breast, and colon (29), epithelial cells are a plausible source of soluble CD44v6 in the circulation. Fig. 5B shows an example of RT-PCR analysis on v6-positive cells microdissected from normal oral mucosa when using a sense primer in exon 5 and an antisense primer in exon 17. Two bands were detected after hybridization with a specific probe of exon v6. First, a broad 1400-bp band was proven to contain amplification products of the variants CD44v2-v10, CD44v3-v10, and CD44v4-v10. The relative differences in the length of these bands are too small to allow them to be resolved on agarose gel. Only when using an antisense primer in exon 11 can they be resolved easily. A detailed survey of the various splice variants expressed in normal oral mucosa and squamous tumors of the head and neck has been published recently (30). The 1000-bp band that is detected has been shown to represent the variant CD44v6-v10. All of these large v6-containing transcripts have been detected in normal as well as malignant squamous cells (30). As shown in Fig. 5C, RT-PCR analysis on v6-positive cells microdissected from normal colon tissue resulted mainly in the same 1000-bp

Fig. 5  RT-PCR was performed on the RNA of (A) nucleated cells from the blood and bone marrow of two non-cancer patients, (B) v6-positive cells microdissected from cryosections of normal oral mucosa from two non-cancer patients (each performed in duplicate), (C) v6-positive cells microdissected from cryosections of normal colon from two non-cancer patients (each performed in duplicate). For patient 2, the blot is rather overexposed; the main fragment visible is the amplification product of CD44v6-v10 (1000 bp). Amplification was performed using a sense primer in exon 5 and an antisense primer in exon 17. Amplification products were hybridized with a specific probe of exon v6.
Soluble CD44v6 in HNSCC

exon 5 sense/exon 17 antisense amplification fragment of the variant CD44v6-v10. These epithelial splice variants might well encode the assortment of domains that appears to be present in the soluble forms in human plasma.

DISCUSSION

Elevated CD44 serum levels have been observed for several tumor types using an exon-specific ELISA (19–24). The high expression of v6-containing CD44 isoforms by HNSCC tumors incited us to investigate the diagnostic value of CD44v6 levels in the circulation of HNSCC patients. Surprisingly, a significant difference between the CD44v6 plasma levels of HNSCC patients, healthy controls, and non-cancer patients could not be observed. In addition, surgical removal of the tumor did not result in a reduction of the CD44v6 plasma level. Notwithstanding these negative results, we could confirm that squamous cancer xenografts do release soluble forms in the circulation of tumor-bearing nude mice. Together, these data indicate that the amount of soluble CD44v6 proteins released from the tumor of HNSCC patients is too small to significantly exceed the basic CD44v6 plasma level. This might be explained by the tumor load of HNSCC patients, which is usually relatively low as compared with other tumor types.

By introducing immunoprecipitation, we expected to enhance the specificity of detection for squamous cell-related isoforms. The basic release of CD44 is thought to have its origin in lymphocytes (16–18), which implies that squamous cell-specific variants might be present in the plasma of HNSCC patients and not in the plasma of non-cancer controls. However, comparison of the spectrum of v6-containing proteins present in the circulation of HNSCC and non-cancer patients did not reveal any changes related to the presence of a tumor.

Subsequently, we investigated whether the release of CD44v6 isoforms expressed by human lymphocytes could explain the observed soluble forms in human plasma. In contrast to what has been published by others, our data show that the spectrum of CD44v6 proteins in normal human plasma cannot be explained by release from lymphocytes or other nucleated blood cells. Immunoprecipitation of v6-containing CD44 proteins from the culture medium of PHA-activated human lymphocytes showed only a low CD44v6 release in vitro. Furthermore, RT-PCR analysis indicated that nucleated cells from blood and bone marrow express mainly a small CD44 isoform which, aside from exon v6, does not contain any other variant exons. It appeared that mRNA transcripts encoding large v6-containing CD44 isoforms are not expressed by nucleated blood cells or are only expressed at a very low level. However, immunoprecipitation of v6-containing proteins from normal human plasma indicated the presence of relatively large v6-containing proteins. Two protein bands of M_r 155,000 and M_r 190,000 were detected in all plasma samples tested. The M_r 190,000 protein could be precipitated with anti-v3, -v5, -v6, and -v9. Therefore, this protein might be the isoform CD44v2-v10 or CD44v3-v10. The M_r 155,000 protein band was only detected after precipitation with anti-CD44s, anti-CD44v6, and anti-CD44v9, and not with anti-v3 or anti-v5, and might therefore represent the protein isoform CD44v6-v10. It should be noted further that some of the bands observed might represent proteolytic fragments or variants with differences in glycosylation, because they contain identical domains (M_r 190,000/M_r 140,000). However, a number of the bands also differ in the assortment of variable domains, whereas the standard domains are present, indicating that these are not related as proteolytic fragments (M_r 190,000/M_r 155,000).

In contrast to nucleated blood cells, large v6-containing CD44 isoforms are highly expressed by for example normal squamous cells. We recently identified the v6-containing mRNA transcripts that are expressed by HNSCC as well as normal oral mucosa by RT-PCR (30). Four highly expressed v6-containing CD44 splice variants could be identified: (a) CD44v2-v10; (b) CD44v3-v10; (c) CD44v4-v10; and (d) CD44v6-v10. In addition to normal oral mucosa, epithelial cells from several other tissues, including the lung and breast, also appear to express these large v6-containing isoforms. Normal epithelial cells of the colon were shown to express mainly the CD44v6-v10 variant. We thus postulate that isoforms CD44v2v10 and CD44v3v10 are released by squamous cells of the mucosal linings and lungs as well as by cells from the breast, whereas the v6-v10 isoform is predominantly released by simple epithelia such as the colon. CD44v6 protein release from the lungs, which have a large contact surface with the blood circulation, are expected to have a particularly large contribution. This hypothesis is underlined by the observation that squamous cancer xenografts do release soluble forms in the circulation of tumor-bearing nude mice. Notwithstanding that these squamous cells are malignant, the expression level and assortment of splice variants are identical between normal and malignant cells. With this hypothesis, we could explain the earlier observation that heavy smokers have a significantly higher plasma level of soluble CD44v6 isoforms (31). Originally, this observation was attributed to activated lymphocytes in the lungs, but the presence of squamous metaplasia or dysplasia of the respiratory epithelium with the concurrent increased v6 expression and v6 release might explain this observation as well. In a larger group
of HNSCC patients \( (n = 30 \); almost all were smokers), we have indeed incidently observed patients \( (n = 3 \) with extremely high soluble CD44v6 plasma levels \( (370–455 \text{ ng/ml}) \), similar to what has been described by Kittl et al. \( (31 \) for smokers (data summarized in Fig. 6). Obviously, these comparisons should be interpreted with caution because the sampling and assays, although identical, were performed in different laboratories. Nevertheless, all these data indicate that there are high levels of CD44v6 variants in normal human plasma, which are released, at least in part, by epithelial cells. These levels severely hamper the use of soluble CD44v6 as a marker of human malignancies.

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Clinical Cancer Research 3541
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