



Soluble CD44 inhibits melanoma tumor growth by blocking cell surface CD44 binding to hyaluronic acid

Thomas Ahrens¹, Jonathan P Sleeman², Christoph M Schempp¹, Norma Howells², Martin Hofmann³, Helmut Ponta², Peter Herrlich² and Jan C Simon^{*1}

¹Department of Dermatology, University of Freiburg, Hauptstrasse 7, D-79104, Freiburg, Germany; ²Forschungszentrum Karlsruhe, Institute of Genetics, D-76021 Karlsruhe, Germany; ³Lion Bioscience AG, Im Neuenheimer Feld 515-517, D-69120 Heidelberg, Germany

Proteolytic cleavage of the extracellular domain of CD44 from the surface of cells has been observed recently in different cell types. In cell culture supernatants of human melanoma cell lines a 70 kDa soluble CD44 protein (solCD44) was detected at concentrations of 250–300 ng/ml. Protease inhibitor studies revealed that serine proteases and metalloproteases are involved in the cleavage of CD44 from the surface of melanoma cells. To analyse a possible function of soluble CD44 a human malignant melanoma cell line was stably transfected with cDNAs encoding either wild type soluble CD44s or mutated forms with defective HA binding properties (CD44sR41A and CD44sR150A/R154A). Soluble CD44s almost completely inhibited hyaluronic acid binding by melanoma cells, whereas soluble CD44 mutated in the HA binding domain had no effect. When cultivated on hyaluronic acid, melanoma cell proliferation was induced by 30% for both the parental and the control transfected cells. This increase in proliferation was blocked completely in solCD44s-secreting transfectants, whereas solCD44sR41A and solCD44sR150A/R154A-secreting cells again showed hyaluronic acid-induced cell proliferation. These cell lines were subcutaneously injected into MF1 nu/nu mice to compare their growth as tumors *in vivo*. Compared to tumors derived from parental and control transfected cells, we observed a dramatic reduction of primary tumor growth with solCD44s expressing MM cells. Transfectants expressing solCD44s mutated in the HA binding domain in contrast developed fast-growing primary tumors. These results provide strong evidence that direct solCD44 interactions with hyaluronic acid interfere competitively with processes induced by hyaluronic acid binding to surface CD44. Autocrine, or drug-induced secretion of solCD44 by human melanoma cells may thus exert potent antitumoral effects *in vivo*. *Oncogene* (2001) 20, 3399–3408.

Keywords: malignant melanoma; hyaluronic acid; CD44; shedding

Introduction

Interactions of cells with components of the extracellular matrix are important for cellular processes like migration, adhesion, proliferation and signal transduction. Hyaluronic acid (HA) is an abundant component of the extracellular matrix. Three receptors for HA on the cell surface, namely CD44 (Aruffo *et al.*, 1990), RHAMM (Turley *et al.*, 1987) and LYVE (Banerji *et al.*, 1999), have been identified. CD44, the principal cellular surface receptor for hyaluronic acid, comprises a family of many isoforms (85–200 kDa) that arise through alternative splicing of the preRNA and different degrees of glycosylation (Naor *et al.*, 1997). CD44s, the standard isoform of the molecule lacking variant epitopes (85–90 kDa) is widely expressed in different tissues and cell types. In addition, CD44 expression is enhanced in a variety of malignant tumors (Naor *et al.*, 1997).

The standard isoform of CD44 binds strongly to hyaluronic acid (Stamenkovic *et al.*, 1991) and amino acid substitutions in the N-terminal part of CD44 led to the characterization of residues which are critical for HA binding (Bajorath *et al.*, 1998; Liao *et al.*, 1995; Peach *et al.*, 1993; Yang *et al.*, 1994). Ligand binding of CD44 has been associated with many cellular processes such as migration, adhesion, signaling and proliferation (Naor *et al.*, 1997). In human melanoma the influence of hyaluronic acid on cell migration has been shown to be CD44 dependent (Thomas *et al.*, 1992) and increased synthesis of hyaluronic acid by melanoma cells is associated with enhanced motility of the cells (Ichikawa *et al.*, 1999). The capacity of HA to induce cell proliferation has been demonstrated by a number of investigators (Greco *et al.*, 1998; West and Kumar, 1989). Evidence for direct CD44-HA interactions resulting in HA stimulated cell proliferation has been provided for eosinophils (Hamann *et al.*, 1995), B-cells (Rafi *et al.*, 1997), T-cells (Galandrini *et al.*, 1994) and keratinocytes (Kaya *et al.*, 1997). For human melanoma cells, HA-induced cell proliferation is completely dependent on CD44-HA interactions (Ahrens *et al.*, 2001).

Modulation of hyaluronic acid synthesis and accumulation around tumor cells has been implicated

*Correspondence: JC Simon

Received 5 January 2001; revised 7 February 2001; accepted 6 March 2001

as playing a critical role in tumor development and metastasis (Itano *et al.*, 1999; Ropponen *et al.*, 1998; Victor *et al.*, 1999; Zhang *et al.*, 1995). In several cases, tumor progression and metastasis formation is mediated by interactions between hyaluronic acid and cell surface CD44 (Peterson *et al.*, 2000; Sy *et al.*, 1991, 1992; Yu *et al.*, 1997). Also for human melanoma, interactions of surface CD44 and hyaluronic acid have been postulated to promote melanoma growth (Bartolazzi *et al.*, 1994).

Proteolytic cleavage of CD44 from the cell surface has been observed in different cell types (Bazil and Horejsi, 1992; Friedl *et al.*, 1997; Goebeler *et al.*, 1996; Gunthert *et al.*, 1996; Okamoto *et al.*, 1999). Increased levels of soluble CD44s and soluble CD44v in the circulation of cancer patients have also been discussed as prognostic markers of disease progression. A correlation of high serum levels of CD44s with tumor burden and metastasis was found in patients with gastric or colon cancer (Guo *et al.*, 1994). High serum levels of soluble CD44 compared to healthy donors were found in patients with ovarian cancer (Zeimet *et al.*, 1997). After subcutaneous transplantation of mammary carcinomas, levels of shedded human CD44 in the serum of mice correlated with tumor size (Fichtner *et al.*, 1997). In the case of melanoma, serum levels of solCD44s and solCD44v were not significantly elevated, although increased levels of solCD44s were detected in the serum of some patients with primary and metastatic malignant melanoma (Schaidler *et al.*, 1997).

The physiological consequences of CD44 shedding and a possible function of the cleaved soluble CD44 protein are not completely understood to date. Migration of a highly metastatic melanoma cell line on immobilized hyaluronic acid was associated with increased CD44s turnover and shedding of the molecule (Goebeler *et al.*, 1996). Shedding of CD44 was also observed in malignant melanoma cells migrating in a 3D-Collagen-Matrix (Friedl *et al.*, 1997), but in a mixed hyaluronic acid-collagen matrix rather integrins than CD44 are the mediators of MM cell migration (Maaser *et al.*, 1999). Finally, a protease inhibitor was able to block the CD44-dependent migration of a lung cancer cell line on a HA coated substrate, indicating a possible role of CD44 shedding during cell migration (Okamoto *et al.*, 1999).

Possible functions of soluble CD44 have also been investigated using recombinant CD44s-Rg fusion proteins. Constant application of a CD44s-Rg fusion protein was able to suppress tumor growth of subcutaneously injected tumor cells in nude mice (Bartolazzi *et al.*, 1994; Sy *et al.*, 1992). Application of a CD44v10-Rg fusion protein prevented metastasis formation in a mouse model, extending possible tumor suppressive functions to epitope v10-containing isoforms of CD44 (Zawadzki *et al.*, 1998). Using another approach, secreted soluble CD44v6-v10 was able to suppress tumor growth of murine mammary carcinoma cells and metastasis formation *in vivo* (Peterson *et al.*, 2000; Yu *et al.*, 1997). Increased apoptosis of soluble

CD44-secreting tumor cells was observed, perhaps caused by interference of soluble CD44 with cell surface CD44-hyaluronic acid interactions (Yu *et al.*, 1997).

In this paper we address the physiological consequences of soluble CD44s release on human melanoma tumor growth *in vivo* and on the proliferative capacity of human MM cells *in vitro*. We demonstrate that competition of cell surface CD44 and soluble CD44 for HA binding sites interferes with HA-dependent processes in these cell lines, suggesting a possible function for soluble CD44. Additionally, we provide evidence for the mechanism underlying CD44 shedding in melanoma cell lines by using protease inhibitors.

Results

Shedding of CD44 in MM cell lines

To investigate the extent to which CD44 is shed from the surface of melanoma cells and whether this has any impact on tumor cell function, we first tested a number of different melanoma cell lines *in vitro* for their ability

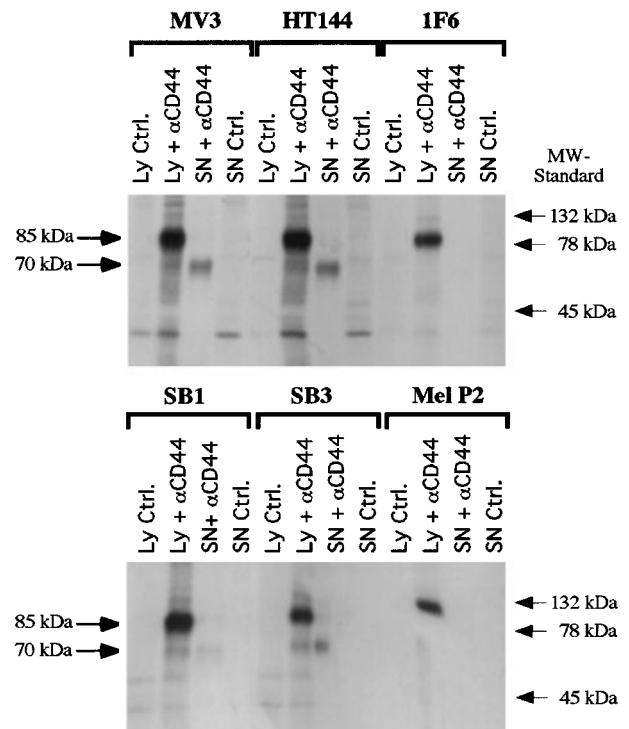


Figure 1 Surface shedding of CD44 in human MM cell lines. 1×10^6 MM cells (MV3, HT144, 1F6, SB1, SB3) and primary melanocytes (Mel P2) were labeled for 48 h with ^{35}S -Methionine/Cysteine. CD44 in total cell lysates (Ly) and cell culture supernatants (SN) was immunoprecipitated with the anti-panCD44 mAb Hermes 3 ($5 \mu\text{g/ml}$) and Protein A/G Sepharose (Ly + αCD44 ; SN + αCD44). As a control, unspecific binding of proteins to Protein A/G Sepharose was included (Ly Ctrl; SN Ctrl). Immunoprecipitated proteins were separated by SDS-PAGE, gels were dried and analysed by autoradiography

to shed CD44 from the cell surface into the cell culture supernatant (Figure 1). Immunoprecipitation of CD44 from radioactively labeled cells was performed to identify CD44 in the protein lysate and the cell culture supernatants. High levels of released CD44 were detected in supernatants of the cell lines MV3 and HT144 (Figure 1; SN+ α CD44), low levels of soluble CD44 were detected in the MM cell lines SB1 and SB3, whereas no soluble CD44 was found in the supernatants of the cell line 1F6 and a primary melanocyte preparation (Figure 1; Mel P2). The apparent molecular weight of the shedded CD44 is 70 kDa, most likely reflecting the truncated form of the 85 kDa membrane-bound CD44s molecule (Figure 1; Ly+ α CD44). Antibodies specific for the variant epitopes CD44v4, CD44v6 and CD44v9 were unable to immunoprecipitate variant CD44 isoforms out of cell lysates and cell culture supernatants (data not shown). We conclude that soluble CD44 from the malignant melanoma cells tested does not contain significant amounts of variant CD44 epitopes and represents the truncated standard part of the molecule lacking the transmembrane and the cytoplasmic domain.

Metalloprotease and serine protease inhibitors completely prevent shedding of CD44 in MM cells

Little is known about the cleavage site of the CD44 protein and the responsible protease. In an attempt to specify proteases involved in CD44 cleavage from the cell surface of MM cells, we performed protease inhibitor studies with the cell lines MV3 and HT144, which shed CD44 (Figure 2). MV3 and HT144 cells were cultured for 6 h and levels of shedded soluble CD44 in the culture supernatant were measured as controls (Figure 2a,b; Medium-Ctrl 6 h). At this time point different protease inhibitors were added for an additional 6 h period. Culture supernatants were then collected again and solCD44 levels were determined using a CD44-ELISA. The effects of protease inhibitors on CD44 shedding were analysed in comparison to solCD44 levels in 12 h culture media from non-treated cells (Figure 2a,b; Medium-Ctrl 12 h). Addition of 2 mM of the metalloprotease inhibitor 1,10-Phenanthroline resulted in a 100% inhibition of CD44 shedding for both MM cell lines. The serine protease inhibitor AEBSF (2 mM) also efficiently blocked the proteolytic cleavage of CD44 in these two MM cell lines (Figure 2a,b). All other tested protease inhibitors, among them Pepstatin A and Phosphoramidon, showed only minor effects on CD44 shedding.

Toxic effects of protease inhibitors on cell viability or effects on protein synthesis in general are often observed in this type of experiment. The kinetics of inhibition of cell surface shedding of CD44 within 6 h compared to the half-life of CD44 on the cell surface which is in the range of 12–20 h (Goebeler *et al.*, 1996; Gunthert *et al.*, 1996) strongly argues for an active process of shedding and inhibition than for an effect of the inhibitors at the level of protein synthesis. To test

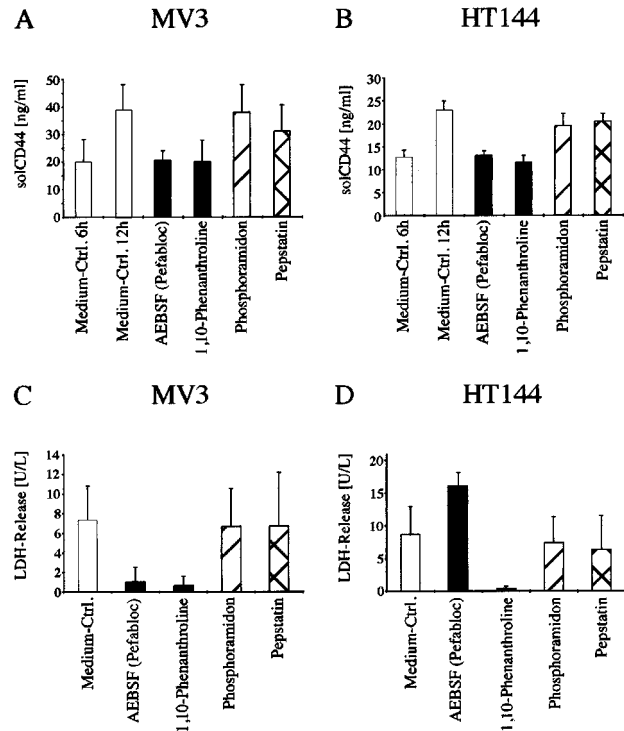


Figure 2 Metalloproteinases and serine proteases are mediators of proteolytic cleavage of CD44. (a) and (b): 5×10^3 MV3 or HT144 MM cells per well were cultivated for 6 h. Soluble CD44 in the supernatant was measured by CD44-ELISA (Medium-Ctrl 6 h). The protease inhibitors AEBSF (2 mM), 1,10-Phenanthroline (2 mM), Phosphoramidon (10 μ M) and Pepstatin A (1 μ M) were then added for a further 6 h. Thereafter soluble CD44 levels in the supernatants were measured again. Non-treated 12 h cell culture supernatants were analysed as a control (Medium-Ctrl 12 h). Data are depicted as mean values \pm s.d. of three independent experiments. (c) and (d): Cytotoxic effects of protease inhibitors were analysed by LDH-Release (Units/Liter) into the cell culture medium. The LDH-Release of non treated cells was determined as control (Medium-Ctrl). Data represent mean values \pm s.d. of three independent experiments

for possible cytotoxic effects of these protease inhibitors we analysed cell viability by measuring LDH-release into the culture medium which is indicative of membrane disruption in dying cells (Figure 2c,d). The addition of 1,10-Phenanthroline and AEBSF did not exert toxic effects on MV3 and HT144 cells when compared to non-treated cells (Figure 2c,d; Medium-Ctrl). Phosphoramidon and Pepstatin were also found to be non toxic for the MM cells. LDH concentrations measured in the cytosol of MM cells in contrast reached 250 U/L (data not shown). In conclusion, metalloproteases and serine proteases are the main mediators of cell surface cleavage of CD44 in MM cells.

Generation of soluble CD44s expressing transfectants of the MM cell line 1F6

A function of native soluble CD44s and its impact on cellular processes like adhesion and proliferation has not been analysed in detail for human malignant

melanoma. To investigate the function of soluble CD44 in a defined cell system, the cell line 1F6 which does not shed CD44 was stably transfected with cDNAs encoding truncated forms of CD44s. The CD44 cDNA used for transfection encodes the standard CD44 exons 1–5, 15 and 16 (Figure 3a; solCD44s), excluding the variant exons v2–10 (Figure 3a; hu CD44-Gene). A TGA translation stop-codon was added to the 3' end of

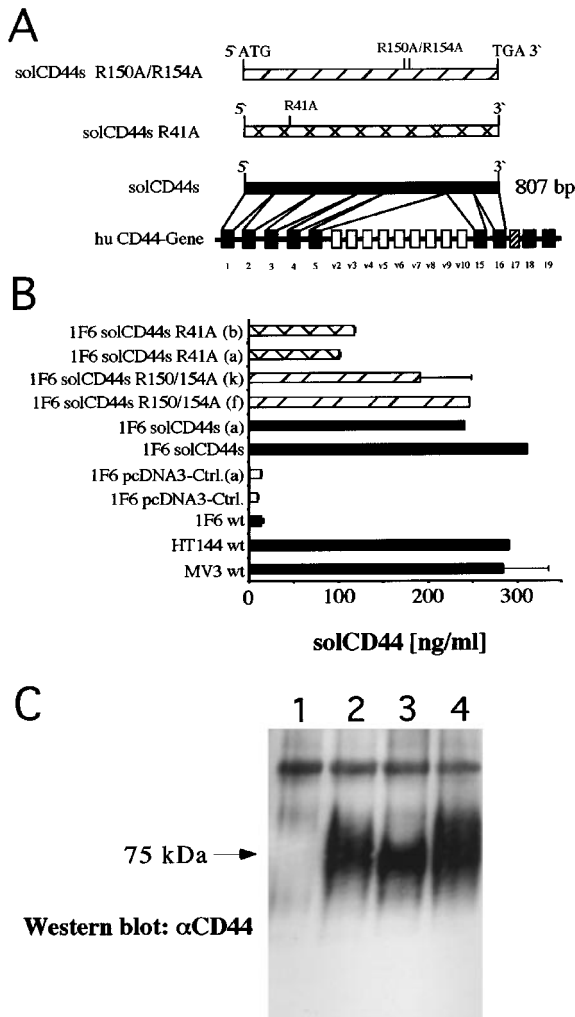


Figure 3 Expression of solCD44s, solCD44sR41A and solCD44sR150/154A in 1F6 melanoma cells. (a): Schematic drawing of the different CD44 cDNAs transfected into 1F6 cells. The complete human CD44 gene structure is depicted (hu CD44-Gene). Alternatively spliced exons v2–10 are marked as grey boxes. The solCD44s cDNA comprises CD44 exons 1–5, 15 and 16 (solCD44s). A TGA stop codon was inserted 3' to the last codon of exon 16. Site directed mutations in codons for R41 (solCD44sR41A) as well as R150 and R154 of CD44 (solCD44sR150/154A) are indicated. (b): 1×10^6 cells of the indicated cell lines were cultivated for 48 h. The supernatants were then collected and soluble CD44 levels were determined by CD44-ELISA. Individual clones were named by letters in parentheses. (c): Western blot of concentrated 48 h cell culture medium of parental 1F6 cells (lane 1) and solCD44sR150/154A (lane 2), solCD44sR41A (lane 3) and solCD44s (lane 4) secreting cells. Two hundred and fifty ng solCD44 were loaded, transferred to PVDF membranes and detected with the anti-panCD44 mAb Hermes 3

the coding sequence of exon 16 resulting in secretion of solCD44s which is devoid of the transmembrane (exon 17) and the cytoplasmic domain (exon 18 or 19).

The cDNA encoding the soluble form of CD44s was point mutated in the codon for amino acid 41, which has been shown to be critical for the HA binding function of CD44 (Peach *et al.*, 1993). This mutation results in an amino acid substitution of arginine 41 to alanine in solCD44s (Figure 3a; solCD44sR41A). Important residues for the HA binding function of CD44 are also arginine 150 and arginine 154 (Liao *et al.*, 1995; Peach *et al.*, 1993). The codons for these two amino acids were also subjected to site directed mutagenesis, resulting in arginine to alanine substitutions (Figure 3a; solCD44sR150A/R154A).

After transfection individual cell clones were analysed for expression of the secreted proteins by CD44-ELISA (Figure 3b). Non-transfected, control-transfected and transfected melanoma cells were cultivated for 48 h, then medium was removed and analysed for soluble CD44 content. Transfection of cDNAs encoding wild type or mutated solCD44s resulted in the release of 125–300 ng CD44 per ml culture medium (Figure 3b). This was equal to the amounts shedded by the non-transfected melanoma cell lines MV3 and HT144. In the culture medium of the parental cell line 1F6 and control-transfectants (Figure 3b; 1F6 pcDNA3-Ctrl) almost no soluble CD44 was detected.

Two hundred and fifty ng soluble CD44 of the different transfectants were analysed by Western blotting to estimate the apparent molecular weights of the secreted proteins (Figure 3c). Soluble CD44s released by the transfected cells (Figure 3c; lanes 2–4) migrated with an apparent molecular weight of 75 kDa which is slightly higher than the naturally cleaved form shown to be approximately a 70 kDa protein (Figure 1). No soluble CD44 was detected in the supernatant of non transfected 1F6 cells (Figure 3c; lane 1).

MM cell adhesion to hyaluronic acid is inhibited by solCD44s

The solCD44s-expressing cell lines were first analysed for surface expression of CD44 and basal proliferation. No significant differences compared to the non-transfected or control-transfected cells were observed (data not shown). To test for competition of soluble CD44s and cell surface CD44 regarding HA binding, we then enriched solCD44s, solCD44sR41A and solCD44sR150/154A from culture supernatants by concentration (Figure 3c).

HA binding competition assays were performed in which for soluble CD44 enriched supernatants competed with MM cells for binding to immobilized HA. Hyaluronic acid-coated 96-well plates were preincubated with the different solCD44s proteins (10 μ g/ml) prior to incubation of the wells with MM cells. Following adhesion of the cells and subsequent washing, the remaining adherent proportion of cells was determined (Figure 4). Unspecific binding was measured as cell adhesion to wells coated with BSA

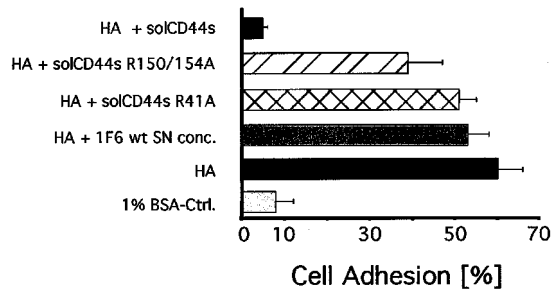


Figure 4 The interference of solCD44s, solCD44sR41A and solCD44sR150/154A with MM cell adhesion to immobilized HA. 5×10^4 cells per well were allowed to adhere on HA coated wells for 30 min at 4°C (HA). As controls for unspecific binding, cells were incubated on BSA coated (1% BSA-Ctrl) wells. The effect of 10 μ g/ml solCD44s, solCD44sR41A or solCD44sR150/154A (HA + solCD44s) on cell adhesion was examined by preincubation of the HA coated wells with the soluble proteins before applying the cells. Cell adhesion was calculated as percentage of adherent cells compared to total number of applied cells (=100%). One representative out of three almost identical experiments is depicted. Data are expressed as mean values of four wells \pm s.d.

(Figure 4; 1% BSA-Ctrl). As a positive control, adhesion of the cells to HA without solCD44 preincubation was determined (Figure 4; HA).

Mutated solCD44sR41A and solCD44sR150/154A did not interfere with binding of MV3 cells to HA (Figure 4). In contrast, non-mutated solCD44s was able to block cell adhesion of MV3 cells to hyaluronic acid almost completely (Figure 4; HA + solCD44s). The concentrated solCD44s negative culture supernatant of the parental 1F6 cells did not show any effect on hyaluronic acid binding (Figure 4; HA + 1F6 wt SN conc) indicating that the effects observed are dependent on soluble CD44. In summary, soluble CD44s is able to compete efficiently with membrane-associated CD44 for HA binding.

Soluble CD44s inhibits hyaluronic acid-induced MM cell proliferation in vitro

There is evidence for a hyaluronic acid dependent increase in proliferation of MM cells. This increase of proliferation is inhibited completely by an anti-CD44 antibody which also blocks binding and adhesion of MM cells to hyaluronic acid (Ahrens et al., 2001). As shown above, the secreted form of CD44 inhibits MM cell adhesion to HA. We reasoned that binding of solCD44s to HA could also result in an inhibition of HA-induced proliferation. To test this hypothesis, MM cells were incubated for 48 h in HA coated and non-coated 96-well plates. [3 H]Thymidine uptake was measured in the non-transfected, the control-transfected and the solCD44s-expressing 1F6 transfectants (Figure 5). The basal proliferation of the different cell lines on uncoated wells was set as 100% proliferation. The basal proliferation of the different transfectants within 48 h was similar ([3 H]Thymidine uptake $4 \times 10^4 \pm 10\%$ counts/min for 2500 cells of each transfectant).

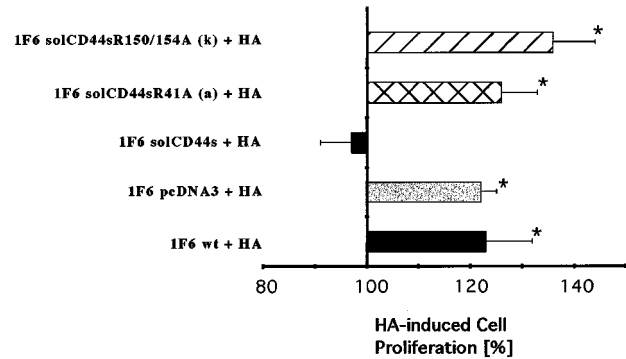


Figure 5 Hyaluronic acid-induced increase of MM cell proliferation is inhibited by solCD44s. 2.5×10^3 1F6 MM cells and transfectants per well were cultured for 48 h on 1.0 mg/ml HA coated or non coated wells. Cells were pulsed directly with 1 μ Ci [3 H]thymidine per well, were harvested after 48 h and [3 H]thymidine incorporation was determined. Data are plotted as percentage decrease or percentage increase of cell proliferation compared to the basal proliferation (=100%) of the different cell lines cultivated without HA. One of three almost identical experiments is shown. Data are expressed as mean values of four wells \pm s.d. Asterisks indicate a statistically significant induction of proliferation (Student's *t*-test, $P < 0.005$) compared to controls without HA.

For the parental cell line 1F6 and the control-transfected cells an increase in proliferation of 25–30% above basal proliferation was observed when they were grown on HA-coated plates (Figure 5; 1F6 wt + HA and 1F6 pcDNA3 + HA). However, soluble CD44s-expressing cells did not display such a hyaluronic acid dependent increase in proliferation (Figure 5; 1F6 solCD44s + HA). Significantly, HA-induced proliferation was nevertheless observed in transfectants expressing solCD44sR41A or solCD44sR150/154A which do not bind HA (Figure 5). We conclude that the soluble CD44s produced by MM cells binds to the immobilized HA and blocks cell surface CD44-mediated interactions with hyaluronic acid. This prevents the cell adhesion and induction of cell proliferation usually initiated by HA-CD44 interactions.

Expression of soluble CD44s results in suppression of melanoma tumor growth in vivo

The above mentioned experiments demonstrate that soluble CD44s interferes with cell surface CD44-mediated processes such as cell adhesion and proliferation of MM cells *in vitro*. To evaluate whether melanoma proliferation and tumor growth is also affected by soluble CD44s *in vivo*, we injected solCD44s-secreting MM cells subcutaneously into MF1 nu/nu mice (Figure 6). As controls, non-transfected 1F6 cells and several mock-transfected 1F6 cell lines were also injected. The parental 1F6 cells (Figure 6; 1F6 wt) and mock-transfected cells (Figure 6; 1F6 pcDNA3) developed fast growing tumors within 10–15 days after injection. Soluble CD44s-secreting transfectants in contrast showed a

dramatic suppression of primary tumor formation after subcutaneous injection (Figure 6; 1F6 solCD44s). Identical results were also obtained after subcutaneous injection of the cell clones 1F6 pcDNA3 (a) and 1F6 solCD44 (a) (Figure 3b and data not shown). This dramatic growth inhibition is dependent on interactions of solCD44s with hyaluronic acid since a solCD44sR41A-secreting transfectant cell line (Figure 7; 1F6 solCD44R41A (a)), where interactions between cell surface CD44 and HA are not disrupted, developed fast growing tumors similar to the parental cells (Figure 7; 1F6). However, tumor growth of solCD44s-R41A secreting cells is delayed. This could be either caused by some residual HA binding capacity of the mutant protein or additional factors contributing to tumor growth delay in human melanoma e.g. apoptosis induction or dysregulation of cell-cell contacts. Thus, the secretion of soluble CD44s efficiently inhibits tumor cell proliferation *in vivo*, extending the results observed *in vitro*. This suppression of tumor development is

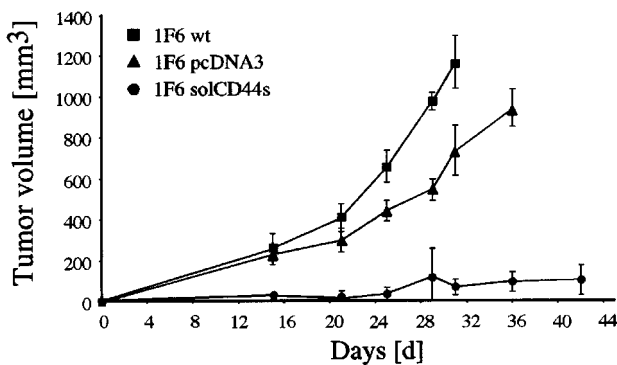


Figure 6 SolCD44s completely suppresses primary tumor growth of MM cells *in vivo*. 5×10^6 cells of the indicated MM cell lines were subcutaneously injected into MF1 nu/nu mice. Animals were monitored daily and the tumor volume was measured every third day. Values represent mean values \pm s.d. of five injected animals per group

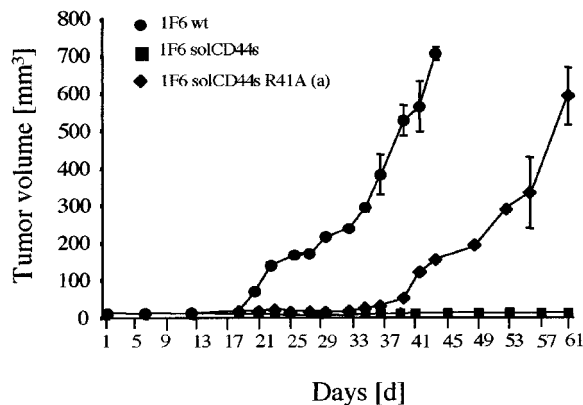


Figure 7 SolCD44sR41A with impaired HA binding function leads to fast developing primary tumors *in vivo*. 5×10^6 cells of the indicated MM cell lines were subcutaneously injected into MF1 nu/nu mice. Animals were monitored each day and the tumor volume was measured every third day. Values represent mean values \pm s.d. of three injected animals per group

critically dependent on the interaction of soluble CD44s with hyaluronic acid. These data suggest that solCD44 interferes with processes which lead to cell proliferation that are usually initiated by the interaction of cell surface CD44 with hyaluronic acid.

Discussion

In this study we describe shedding of the hyaluronic acid receptor CD44 from the surface of melanoma cells which is caused by proteolytic cleavage through metallo- and serine proteases. Enforced expression of secreted soluble CD44s completely abolished melanoma cell adhesion to immobilized HA and in addition inhibited the hyaluronic acid-induced proliferation of melanoma cells. In contrast, soluble CD44s with defective HA binding properties did not interfere with cell adhesion to hyaluronic acid and did not inhibit HA-induced proliferation. *In vivo*, a dramatic reduction in tumor growth was observed after subcutaneous injection of soluble CD44s-secreting cells into nu/nu mice. However, melanoma cells secreting soluble CD44s unable to bind to HA showed formation of primary tumors which was slightly retarded but still similar to the tumor development of non-transfected parental cells.

Proteolytic cleavage of membrane proteins is a common mechanism which regulates receptor expression on the cell surface (Bazil, 1995). Shedding of adhesion molecules and involvement of proteases in the cleavage process has been previously reported for L-Selectin (Chen *et al.*, 1995), VCAM (Leca *et al.*, 1995) and ICAM-1 (Champagne *et al.*, 1998). Metalloproteases have been associated with the cleavage of CD44 in TPA stimulated granulocytes (Bazil and Strominger, 1994) and a lung cancer cell line (Okamoto *et al.*, 1999). Using a variety of protease inhibitors we clearly identified metalloproteases and serine proteases as being involved in the cleavage of CD44 in melanoma cells, whereas all other tested protease inhibitors only showed minor effects on CD44 shedding. To date, the proteolytic activity responsible for CD44 shedding has not been assigned to a specific protease.

The apparent molecular weight of shedded CD44 in MM cells is around 70 kDa, which is in agreement with the expected size for the extracellular, glycosylated part of CD44s. Variant epitopes were not detected in the soluble CD44 using a panel of different anti-variant CD44 mAb, which is also consistent with the shedded CD44 being the extracellular portion of CD44s. Although the exact cleavage site of membrane-bound CD44 has not been identified, deletion of the membrane proximal domains of CD44 encoded by exons 15 and 16 does not abolish cleavage of CD44 (Okamoto *et al.*, 1999), which argues for a rather undefined cleavage site.

Increased tumor cell proliferation and metastasis formation has been associated with CD44-HA interactions (Bartolazzi *et al.*, 1994, 1995; Sy *et al.*, 1991, 1992). Furthermore, enhanced synthesis of hyaluronic

acid by tumor cells has been proven to promote tumor growth (Kosaki *et al.*, 1999) and metastasis (Itano *et al.*, 1999; Zhang *et al.*, 1995), and has also been observed in highly metastatic MM cell lines (Goebeler *et al.*, 1996). Moreover, an influence of soluble CD44 on cell migration, tumor development and metastasis formation by melanoma cells has also been reported (Bartolazzi *et al.*, 1994; Thomas *et al.*, 1992; Zawadzki *et al.*, 1998), and most likely functions by interfering with cell surface CD44-HA interactions. Thus, soluble CD44 could have a major impact on melanoma tumor growth *in vivo*. The dramatic inhibition of primary tumor formation we observed for solCD44s-expressing MM cells is consistent with this notion. Tumor growth arrest was critically dependent on soluble CD44-HA interactions because soluble CD44s with impaired HA binding functions interfered with tumor development to a much smaller extent. The small effect of mutated soluble CD44s on tumor growth is consistent with our cell adhesion experiments in which the mutated soluble CD44 proteins displayed only small inhibiting effects on cell adhesion, perhaps due to residual HA binding capacity of the mutant proteins.

Our data suggest that the HA binding function of CD44s plays a critical role in the growth of melanoma tumors, and that this function can be regulated by shedding of CD44s. In several experimental systems HA binding by soluble CD44 was found to be critical for the growth and metastasis inhibiting effects observed (Bartolazzi *et al.*, 1994; Peterson *et al.*, 2000; Sy *et al.*, 1992; Yu *et al.*, 1997). We also found that an intact HA binding function of soluble CD44 is critical for competition with cell surface CD44. Competition of solCD44s with membrane-bound CD44 for HA binding sites could interfere with cellular processes usually initiated upon HA binding of cells. Here and in previous work (Ahrens *et al.*, 2001) we have demonstrated that hyaluronic acid stimulates melanoma proliferation in a concentration- and CD44-dependent manner. This proliferation-inducing effect is blocked via release of soluble CD44 which still retains its HA binding capacity. This extends the effects of soluble CD44 that have been reported in the context of cell migration (Okamoto *et al.*, 1999; Thomas *et al.*, 1992) to cell proliferation of melanoma cells. Release of the growth factors bFGF and TGF- β 1 are associated with the culture of MM cells on hyaluronic acid (Ahrens *et al.*, 2001). These results suggest an autocrine loop mechanism of proliferation induction via CD44-HA interactions and subsequent growth factor release.

In culture, soluble HA-binding CD44s was able to reduce proliferation of HA-treated melanoma cells to the proliferation levels observed in the absence of HA. However, *in vivo*, soluble HA-binding CD44s dramatically inhibited tumor growth. These data would suggest that MM tumor growth critically depends on HA-induced proliferation, perhaps through the autocrine stimulation we have observed in culture. Alternatively or in addition, solCD44s could have other functions. For example, it has been shown that

increased cell death was found in a mammary carcinoma cell line as a result of solCD44v6-v10 secretion (Yu *et al.*, 1997), and solCD44s may act analogously in the case of MM.

The influence of hyaluronic acid-CD44 interactions in the progression of gastric, colon, ovarian and breast cancer where poor prognosis is often associated with the expression of the CD44v6 epitope have not been addressed in detail. Variant CD44v6 has been shown to promote metastasis formation independent of interactions with hyaluronic acid (Sleeman *et al.*, 1996). In the case of melanoma the influence of variant CD44v6 expression on the metastatic potential of the cells seems to be rather low (Simon *et al.*, 1996). Overexpression of cell surface CD44 in primary melanoma instead is often correlated with high risk of metastasis formation (Dietrich *et al.*, 1997), consistent with a critical role for CD44s in promoting tumor growth through ligating HA.

Elevated levels of soluble CD44 has been reported in the serum of patients with a variety of carcinomas (Guo *et al.*, 1994; Lackner *et al.*, 1998; Martin *et al.*, 1997; Zeimet *et al.*, 1997). In contrast, no significant elevation was found in sera from melanoma patients, although some primary and metastatic malignant melanoma patients showed high levels of solCD44s (Schaidler *et al.*, 1997). Our data would suggest that high serum levels of solCD44s might be a positive prognostic factor for malignant melanoma patients. We therefore concur with the conclusion of Schaidler *et al.* (1997) that malignant melanoma patients with high serum levels of solCD44s should be studied in more detail.

Finally, our results may have important clinical applications. We show that growth promoting signals in MM cells usually induced by direct interaction of cell surface CD44 with hyaluronic acid are efficiently competed and inhibited by soluble CD44s. The loss of adhesive contacts between MM cells and hyaluronic acid results in a marked decrease in MM cell proliferation *in vitro* and tumor growth *in vivo*. Thus, disruption of receptor-ligand interactions by use of soluble forms of receptors may represent a possible target for antitumor therapy.

Materials and methods

Media and Reagents

MM cells were cultured in RPMI 1640 (Seromed, Eching, Germany) supplemented with penicillin G (50 U/ml, Gibco, Eggenstein, Germany), streptomycin (50 μ g/ml, Gibco), 2 mM L-glutamine (Gibco) and 10% heat-inactivated fetal calf serum (PAA, Coelbe, Germany). Cultures of non-transformed melanocytes were maintained in selective melanocyte growth medium MGM (Promo Cell, Heidelberg, Germany). Rooster comb hyaluronic acid (Sigma, Deisenhofen, Germany) was used for HA-binding studies and proliferation assays. The CD44s ELISA-Kit (Bender Med Systems, Vienna, Austria) for the determination of solCD44 levels in MM cell culture supernatants was used according to

the manufacturer's instructions. The following protease inhibitors were used: AEBSF in H₂O (Boehringer Mannheim, Mannheim, Germany); 1,10-Phenanthroline in MeOH (Sigma); Pepstatin A in DMSO (Sigma) and Phosphoramidon in H₂O (Sigma).

Cell lines and stable transfections

The MM cell line HT144 was obtained from the ATCC (Rockville, MD, USA), MV3 and 1F6 were a gift of Dr GP van Muijen (Dept. of Pathology, Nijmegen, The Netherlands), SB1 and SB3 were a gift of Dr CV Hamby (New York Medical College, Valhalla, NY, USA). Cultures of non-transformed melanocytes were established from human neonatal foreskin as described (Simon *et al.*, 1996). Purity (>95%) of the melanocyte cultures was confirmed by FACS-staining with the melanocyte-specific mAbs HMB-45 and S100. Melanocyte cultures between passages 2 and 5 (P2 to P5) were used for the experiments. Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. To generate stable transfectants 5 × 10⁶ MM cells were resuspended in 500 μl medium containing 20 μg plasmid DNA. Cells were electroporated (950 μF, 250 V) in 0.4 mm electroporation cuvettes (BioRad, Muenchen, Germany) using a GenePulser (BioRad, Muenchen, Germany). After addition of 1 ml medium, cells were incubated for 10 min on ice. Cells were selected in medium containing 1 mg/ml G418 (Gibco). Resistant clones were picked 2–3 weeks after application of the selection medium. Expression of the cDNAs was assessed by both Western blotting and CD44-ELISA analysis of cell culture supernatants.

Monoclonal antibodies

Following monoclonal antibodies were used: mlgG₁ control mAbs were obtained from Becton Dickinson (Heidelberg, Germany), anti-panCD44 (Hermes 3, mlgG₁) was a generous gift of Dr S Jalkanen (University of Turku, Finland), rat anti mouse CD44 mAb IM7 was purchased from Pharmingen (Pharmingen, Hamburg, Germany), HRP-conjugated goat anti-mouse IgG was obtained from DAKO (Hamburg, Germany).

Protease inhibitor studies

5 × 10³ melanoma cells per well were cultivated for 6 h on 96-well plates (Falcon, Heidelberg, Germany). After 6 h the level of CD44 shed into the cell culture supernatant was determined by CD44-ELISA (Bender Med Systems, Vienna, Austria). The protease inhibitors 1,10-Phenanthroline (2 mM), AEBSF (2 mM), Pepstatin A (1 μM) and Phosphoramidon (10 μM) were added in non toxic concentrations. Cells were then incubated for an additional 6 h at 37°C. Cell culture supernatants were collected and solCD44 concentrations in the medium were measured by CD44-ELISA. As a control, non-treated 12 h supernatants were analysed. Data are depicted as mean values ± s.d. of three independent experiments.

Cytotoxicity assay

Plasma membrane permeability of MM cells was assessed by Lactate dehydrogenase (LDH) release of the cells into the cell culture medium after 6 h of coculture with protease inhibitors. LDH was measured by a coupled enzymatic reaction using the Cobas Mira Plus system (Roche, Grenzach, Germany). Data are expressed as Units/Liter of mean values ± s.d. of three independent experiments.

solCD44 cDNA construction and site directed mutagenesis

A truncated human CD44s cDNA encoding exons 1–5, 15 and 16 (Screaton *et al.*, 1992), modified by a 3' terminal TGA-stop codon, was cloned into the *KpnI* and *EcoRV* sites of the pcDNA3-Vector (Invitrogen, Groningen, The Netherlands). Mutated soluble CD44s expression constructs encoding solCD44sR41A and solCD44sR150A/R154A (Liao *et al.*, 1995; Peach *et al.*, 1993) were generated using the Quik Change™ site-directed mutagenesis kit (Stratagene, Heidelberg, Germany). In brief, 20 ng of the solCD44s expression vector were PCR amplified (94°C for 30 s, 55°C for 1 min, 68°C for 14 min; 16 cycles) with *Pfu* DNA polymerase using sense and antisense oligonucleotides containing the R41A or R150A/R154A mutations, respectively. Oligonucleotides used, were: R41A sense: 5'-CACGTGGAGAAAAATGGC-GCCTACAGCATCTCTCGG-3'; R41A antisense: 5'-CC-GAGAGATGCTGTAGGCGCCATTTTTCTCCACGTG-3'; R150A/R154A sense: 5'-CTATTGTCAACGCTGATGGCA-CCGCCTATGTCCAG-3'; R150A/R154A antisense: 5'-CT-GGACATAGGCGGTGCCATCAGCGTTGACAATAG-3'. The non-mutated parental supercoiled dsDNA was subsequently digested with 1 U *DpnI* After transformation of XL1-blue Supercompetent Cells, plasmid DNA was purified and analysed by sequencing.

Enrichment of solCD44 from cell culture supernatants

Supernatants of transfectants grown for 48 h in expression medium containing 1% FCS were collected and concentrated using Centricon 100 columns with a molecular weight cut off 30 kDa (Amicon, Beverly, MA, USA). After dialysis against PBS w/o Mg²⁺/Ca²⁺, the solCD44 concentration was determined using a CD44-ELISA. For the subsequent HA binding assays, soluble CD44 was applied at a concentration of 10 μg/ml to HA coated plastic surfaces.

Western blot analysis

Two hundred and fifty ng solCD44 were diluted in 1 ml lysis buffer (PBS containing 0.5% NP-40 and 2 mM PMSF (Sigma)) and immunoprecipitated overnight at 4°C with 5 μg/ml rat anti mouse CD44 mAb IM7 (Pharmingen, Hamburg, Germany) and 50 μl Protein A/G Sepharose beads (Calbiochem, Bad Soden, Germany). After centrifugation at 14 000 r.p.m. for 4 min at 4°C the immunoprecipitates were washed three times resolved in 2 × non reducing Laemmli-buffer, boiled for 5 min and separated by SDS-PAGE (8% polyacrylamide gel). After centrifugation at 14 000 r.p.m. for 10 min, 25 μg of the total cell lysate were separated by SDS-PAGE. Proteins were electrically transferred to PVDF membranes (Millipore, Eschborn, Germany). Detection of CD44 was performed using the anti human CD44 mAb Hermes 3 (5 μg/ml). Protein bands were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham, Braunschweig, Germany).

Metabolic labeling and immunoprecipitation

5 × 10⁵ MM cells were cultured on 10 cm culture dishes for 24 h. Cells were then starved for 2 h in cRPMI (RPMI 1640 w/o methionine and cysteine; 10% dialyzed FCS; 2 mM L-glutamine, L-lysine and L-leucine). Afterwards CD44 was cleaved from the cell surface for 15 min with 1 ml 0.25% trypsin. Cells were replated and labeled for 48 h with 750 μCi L-³⁵S-methionine/cysteine (Amersham) per dish. Supernatants were collected and centrifuged (14 000 r.p.m., 3 min, 4°C).

Cells were lysed in 1 ml RIPA-buffer and also centrifuged. Cell lysates and respective supernatants were precleared overnight with 100 μ l Protein A/G Sepharose beads. CD44 was then immunoprecipitated for 2 h at 4°C with anti-panCD44 mAb Hermes 3 (5 μ g/ml) and Protein A/G Sepharose beads. Immunoprecipitates were washed four times with RIPA-buffer and after boiling the samples at 90°C for 5 min the immunoprecipitated proteins were separated by SDS-PAGE. After incubation in PPO (20% w/v in DMSO) the gel was dried and analysed by autoradiography.

Cell adhesion assays

Hyaluronic acid (1 mg/ml in 50 mM NaHCO₃-buffer, pH 9.6) was coated overnight on 96-well flat bottom plates (Greiner, Frickenhausen, Germany) at 4°C. Plates were then washed three times with PBS w/o Ca²⁺Mg²⁺. To avoid unspecific binding, plates were subsequently coated with 1% (w/v) heat denatured BSA (Sigma) in PBS for 1 h at 37°C and washed again with PBS. Ten μ g/ml of solCD44, solCD44sR41A and solCD44sR150A/R154A of different MM cell lines were then added to the 96-well plates for 2 h at 4°C. 5 × 10⁴ [³H]thymidine (Amersham) prelabeled cells were seeded per well and plates were incubated for 45 min at 4°C. After rinsing the wells three times with PBS, remaining adherent cells were harvested and counted using a liquid scintillation β -counter (Top-Count, Canberra Packard, Dreieich, Germany). As a control, adhesion to 1% (w/v) BSA coated wells was measured. The percentage of cell adhesion was determined as ratio of counts/min of remaining adherent cells to counts/min of the 5 × 10⁴ initially applied cells.

Proliferation assays

Ninety-six-well flat bottom plates (Falcon) were coated overnight at 4°C with 100 μ l heat inactivated (20 min at 100°C) hyaluronic acid (1.0 mg/ml in 50 mM NaHCO₃-buffer,

pH 9.6). After rinsing the wells three times with PBS, 2.5 × 10³ cells of the different MM cell lines were seeded per well. To measure cell proliferation, 1 μ Ci [³H]thymidine per well was added. After a total of 48 h cells were harvested and [³H]thymidine uptake was determined using the Top-Count β -counter (Canberra Packard). Statistical analysis of data was performed applying Student's *t*-test.

Injection of tumor cells in vivo and monitoring of tumor growth

Pathogen free melanoma cells were resuspended in PBS w/o Ca²⁺/Mg²⁺ and 5 × 10⁶ cells in a volume of 0.1 ml were injected subcutaneously into the flank of each MF1 nu/nu mouse (Jackson Laboratory, Bar Harbor, USA). Five animals per group were injected with melanoma cells. The animals were monitored daily and tumor volume was measured every second day using a venier calliper. The mice were usually killed when the tumor volume reached approximately 1000 mm³.

Abbreviations

HA, hyaluronic acid; ECM, extracellular matrix; MM, malignant melanoma; solCD44, soluble CD44; CD44s, standard CD44; CD44v, variant CD44; SN, supernatant

Acknowledgments

We thank Brigitte Mai, Anke Stingl and Ursula Voith for excellent technical assistance. In addition, we thank CV Hamby for kindly providing us with the SB1 and SB3 melanoma cell lines and GN van Muijen for sending us the MV3 and 1F6 melanoma cell lines. This work was supported by grant He 551/8 from the Deutsche Forschungsgemeinschaft.

References

- Ahrens T, Assmann V, Fieber C, Termeer CC, Herrlich P, Hofmann M and Simon JC. (2001). *J. Invest. Dermatol.*, **116**, 93–101.
- Aruffo A, Stamenkovic I, Melnick M, Underhill CB and Seed B. (1990). *Cell*, **61**, 1303–1313.
- Bajorath J, Greenfield B, Munro SB, Day AJ and Aruffo A. (1998). *J. Biol. Chem.*, **273**, 338–343.
- Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, Jones M and Jackson DG. (1999). *J. Cell. Biol.*, **144**, 789–801.
- Bartolazzi A, Jackson D, Bennett K, Aruffo A, Dickinson R, Shields J, Whittle N and Stamenkovic I. (1995). *J. Cell. Sci.*, **108**, 1723–1733.
- Bartolazzi A, Peach R, Aruffo A and Stamenkovic I. (1994). *J. Exp. Med.*, **180**, 53–66.
- Bazil V. (1995). *Immunol. Today*, **16**, 135–140.
- Bazil V and Horejsi V. (1992). *J. Immunol.*, **149**, 747–753.
- Bazil V and Strominger JL. (1994). *J. Immunol.*, **152**, 1314–1322.
- Champagne B, Tremblay P, Cantin A and St. Pierre Y. (1998). *J. Immunol.*, **161**, 6398–6405.
- Chen A, Engel P and Tedder TF. (1995). *J. Exp. Med.*, **182**, 519–530.
- Dietrich A, Tanczos E, Vanscheidt W, Schopf E and Simon JC. (1997). *Eur. J. Cancer*, **33**, 926–930.
- Fichtner I, Dehmel A, Naundorf H and Finke LH. (1997). *Anticancer Res.*, **17**, 3633–3645.
- Friedl P, Maaser K, Klein CE, Niggemann B, Krohne G and Zanker KS. (1997). *Cancer Res.*, **57**, 2061–2070.
- Galandrini R, Galluzzo E, Albi N, Grossi CE and Velardi A. (1994). *J. Immunol.*, **153**, 21–31.
- Goebeler M, Kaufmann D, Brocker EB and Klein CE. (1996). *J. Cell. Sci.*, **109**, 1957–1964.
- Greco RM, Iocono JA and Ehrlich HP. (1998). *J. Cell. Physiol.*, **177**, 465–473.
- Gunthert AR, Strater J, von Reyher U, Henne C, Joos S, Koretz K, Moldenhauer G, Krammer PH and Moller P. (1996). *J. Cell. Biol.*, **134**, 1089–1096.
- Guo YJ, Liu G, Wang X, Jin D, Wu M, Ma J and Sy MS. (1994). *Cancer Res.*, **54**, 422–426.
- Hamann KJ, Dowling TL, Neeley SP, Grant JA and Leff AR. (1995). *J. Immunol.*, **154**, 4073–4080.
- Ichikawa T, Itano N, Sawai T, Kimata K, Koganehira Y, Saida T and Taniguchi S. (1999). *J. Invest. Dermatol.*, **113**, 935–939.
- Itano N, Sawai T, Miyaishi O and Kimata K. (1999). *Cancer Res.*, **59**, 2499–2504.
- Kaya G, Rodriguez I, Jorcano JL, Vassalli P and Stamenkovic I. (1997). *Genes Dev.*, **11**, 996–1007.
- Kosaki R, Watanabe K and Yamaguchi Y. (1999). *Cancer Res.*, **59**, 1141–1145.

- Lackner C, Moser R, Bauernhofer T, Wilders-Truschnig M, Samonigg H, Berghold A and Zatloukal K. (1998). *Breast Cancer Res. Treat.*, **47**, 29–40.
- Leca G, Mansur SE and Bensussan A. (1995). *J. Immunol.*, **154**, 1069–1077.
- Liao HX, Lee DM, Levesque MC and Haynes BF. (1995). *J. Immunol.*, **155**, 3938–3945.
- Maaser K, Wolf K, Klein CE, Niggemann B, Zanker KS, Brocker EB and Friedl P. (1999). *Mol. Biol. Cell.*, **10**, 3067–3079.
- Martin S, Jansen F, Bokelmann J and Kolb H. (1997). *Int. J. Cancer*, **74**, 443–445.
- Naor D, Sionov RV and Ish-Shalom D. (1997). *Adv. Cancer Res.*, **71**, 241–319.
- Okamoto I, Kawano Y, Tsuiki H, Sasaki J, Nakao M, Matsumoto M, Suga M, Ando M, Nakajima M and Saya H. (1999). *Oncogene*, **18**, 1435–1446.
- Peach RJ, Hollenbaugh D, Stamenkovic I and Aruffo A. (1993). *J. Cell. Biol.*, **122**, 257–264.
- Peterson RM, Yu Q, Stamenkovic I and Toole BP. (2000). *Am. J. Pathol.*, **156**, 2159–2167.
- Rafi A, Nagarkatti M and Nagarkatti PS. (1997). *Blood*, **89**, 2901–2908.
- Ropponen K, Tammi M, Parkkinen J, Eskelinen M, Tammi R, Lipponen P, Agren U, Alhava E and Kosma VM. (1998). *Cancer Res.*, **58**, 342–347.
- Schaider H, Rech-Weichselbraun I, Richtig E, Seidl H, Soyer HP, Smolle J and Kerl H. (1997). *J. Am. Acad. Dermatol.*, **36**, 209–213.
- Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U and Bell JI. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 12160–12164.
- Simon JC, Heider KH, Dietrich A, Wuttig C, Schopf E, Adolf GR, Ponta H and Herrlich P. (1996). *Eur. J. Cancer*, **32A**, 1394–1400.
- Sleeman JP, Arming S, Moll JF, Hekele A, Rudy W, Sherman LS, Kreil G, Ponta H and Herrlich P. (1996). *Cancer Res.*, **56**, 3134–3141.
- Stamenkovic I, Aruffo A, Amiot M and Seed B. (1991). *EMBO J.*, **10**, 343–348.
- Sy MS, Guo YJ and Stamenkovic I. (1991). *J. Exp. Med.*, **174**, 859–866.
- Sy MS, Guo YJ and Stamenkovic I. (1992). *J. Exp. Med.*, **176**, 623–627.
- Thomas L, Byers HR, Vink J and Stamenkovic I. (1992). *J. Cell. Biol.*, **118**, 971–977.
- Turley EA, Moore D and Hayden LJ. (1987). *Biochemistry*, **26**, 2997–3005.
- Victor R, Chauzy C, Girard N, Gioanni J, d'Anjou J, Stora De Novion H and Delpesch B. (1999). *Int. J. Cancer*, **82**, 77–83.
- West DC and Kumar S. (1989). *Exp. Cell. Res.*, **183**, 179–196.
- Yang B, Yang BL, Savani RC and Turley EA. (1994). *EMBO J.*, **13**, 286–296.
- Yu Q, Toole BP and Stamenkovic I. (1997). *J. Exp. Med.*, **186**, 1985–1996.
- Zawadzki V, Perschl A, Rosel M, Hekele A and Zoller M. (1998). *Int. J. Cancer*, **75**, 919–924.
- Zeimet AG, Widschwendter M, Uhl-Steidl M, Muller-Holzner E, Daxenbichler G, Marth C and Dapunt O. (1997). *Br. J. Cancer*, **76**, 1646–1651.
- Zhang L, Underhill CB and Chen L. (1995). *Cancer Res.*, **55**, 428–433.