

# Platelet-Endothelial Cell Adhesion Molecule-1 (CD31) Recycles and Induces Cell Growth Inhibition on Human Tumor Cell Lines

Viviana P. Lutzky,<sup>1</sup> Romina P. Carnevale,<sup>1</sup> Mariano J. Alvarez,<sup>2</sup> Paulo Maffia,<sup>1</sup> Sandra I. Zittermann,<sup>1,3</sup> Osvaldo L. Podhajcer,<sup>2</sup> Andrew C. Issekutz,<sup>3</sup> and H. Eduardo Chuluyan<sup>1\*</sup>

<sup>1</sup>Laboratorio de Inmunogenética, Hospital de Clínicas “José de San Martín”, Facultad de Medicina, Universidad de Buenos Aires, Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina

<sup>2</sup>Instituto Leloir, CONICET, Universidad de Buenos Aires, Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina

<sup>3</sup>Dalhousie University, Halifax, Nova Scotia, Canada

**Abstract** CD31 (PECAM-1) is a 130-kDa member of the immunoglobulin gene superfamily expressed on endothelial cells, platelets, and most leukocytes. This report demonstrates by Western Blot and immunofluorescence that some human melanoma and adenocarcinoma cell lines express CD31 on the cell surface. The surface expression of CD31 was regulated by cell–cell contact, being higher on sparse and spontaneously detached cells. Indeed, fixing and permeabilizing tumor cells revealed a cytoplasmic pool, which was confirmed by confocal microscopy. Some of the plasma surface molecule is endocytosed following mAb binding. Engagement of CD31 on tumor cells via domain-3 inhibited proliferation by inducing cell apoptosis. On the other hand, apoptosis does not increase CD31 expression. Overall, these results indicate that there is an intracellular pool of CD31 on some tumor cells, which modulates CD31 surface expression and its role in cancer cell growth and metastasis. Thus, the expression of CD31 and its role in cell survival in some tumor cells appears to differ from endothelial cells. *J. Cell. Biochem.* 9999: 1–17, 2005. © 2005 Wiley-Liss, Inc.

**Key words:** PECAM-1; CD31; melanoma; adenocarcinoma; cell growth; apoptosis

Metastasis is an important feature of human cancer and this property contributes to poor prognosis. The mechanisms of tumor invasion and acquisition of capability to metastasize remain unclear. Metastasis depends in part on the adhesion properties of neoplastic cells. Decreased adhesiveness of individual or small

groups of cells from primary tumor contributes to the metastatic process. A second step involves the adhesion of circulating tumor cells to vascular endothelium and subendothelial matrix. These cell–cell and cell–matrix interactions involve cell surface glycoprotein receptors [Orr et al., 2000]. Five major protein families have been implicated in mediating cell–cell interactions. They are integrins, cadherins, immunoglobulin superfamily (IgSF), selectins, and mucins. Among them, carbohydrate or carbohydrate–selectin interactions occur at an early stage of the metastatic cascade. Subsequent steps of cell adhesion require integrin or IgSF-based adhesion [Johnson, 1999]. Emerging evidence points to the importance of adhesion molecules leading to the generation of junctional complexes like tight junctions and adherens junctions which regulate both endothelial interactions and paracellular permeability [Santoso et al., 2005].

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\*Correspondence to: H. Eduardo Chuluyan, Laboratorio de Inmunogenética, Piso 3, Sala 4, Hospital de Clínicas “José de San Martín”, Facultad de Medicina, Universidad de Buenos Aires, Avenida Córdoba 2351, (C.P. 1120), Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina. E-mail: chulu@interar.com.ar

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Furthermore, cytokine and chemokine gradients are central to the directed movement of cells. Thus, chemokine receptor expression and activation on malignant cells are important to the growth, survival, and migration of cancer cells from the primary tumor [Kulbe et al., 2004]. The cellular and molecular steps required for metastasis are similar for all cancer cells. These steps are influenced by the stromal microenvironment, which mediates in large part, by bi-directional interactions between tumor cells and neighboring stromal cells, such as fibroblast as well as endothelial and immune cells [Bogenrieder and Herlyn, 2003].

CD31 (PECAM-1) is a 130-kDa member of the IgSF that is expressed on endothelial cells, platelets, and most leukocytes [Albelda et al., 1990; Newman, 1997]. CD31 concentrates at the junctions of endothelial cells in all vessel types. It contains six Ig-like domains, a short transmembrane domain, a long cytoplasmic tail with alternative splicing of exons 10–16 with potential sites for phosphorylation and post-translational modifications [Newman et al., 1990]. Previous reports have indicated that CD31 can engage in both homophilic and heterophilic interactions with other cell surface molecules [Newton et al., 1997]. The heterophilic ligands proposed include a molecule expressed by parasitized red blood cells [Treutiger et al., 1997], the ADP-ribosyl cyclase CD38 [Deaglio et al., 1998], and a CD31 ligand on T cells [Prager et al., 1996].

CD31 mediates a range of different functions, among them recruitment of leukocytes to inflammatory sites [Liao et al., 1997], vasculogenesis [Pinter et al., 1997], angiogenesis [DeLisser et al., 1997], and regulation of monocyte, neutrophil, and T cell activation [Elias et al., 1998; Newman, 1999]. Tyrosine and threonine residues in the cytoplasmic tail of CD31 are phosphorylated leading to the recruitment of cytoplasmic signaling and adaptor molecules including the phosphatases SHP-1 and SHP-2 as well  $\alpha$ - and  $\beta$ -catenin. Furthermore, CD31 has also been assigned to the immunoreceptor tyrosine-based inhibitory motif (ITIM) superfamily, since it modulates the immunoreceptor tyrosine-based activation motif-dependent signaling cascade [Newman, 1999]. Recent studies have demonstrated an even wider range of functions for CD31 including maintenance of adherens junction integrity and permeability, organization of the cytoskeleton, trans-

criptional activities, participation in STAT isoform signaling among others [Ilan and Madri, 2003].

Contrary to the role of CD31 in leukocyte migration, its role in tumor metastasis remains less clear. Recently, Voura et al. [2000] showed that endothelial CD31 is not required for CD31<sup>-</sup> melanoma cell transendothelial migration. However, previously Tang et al. [1993] identified CD31<sup>+</sup> melanoma and adenocarcinoma cell lines and showed a role in cell adhesion to endothelium. In some tumor tissue fragments, the membrane expression of CD31 was rare, though weak cytoplasmic staining was seen in some cases [De Young et al., 1998]. Furthermore, CD31 expression was observed in a breast carcinoma [Sapino et al., 2001]. In the present work, we analyzed the expression of CD31 on melanoma and adenocarcinoma cell lines, in order to examine the putative factors or mechanisms that modulate its surface expression. Furthermore, we also analyzed the role of CD31 on tumor cell proliferation and cell apoptosis. This is particularly interesting for tumor physiology, due to the recently described role of CD31 in the transduction of a survival signal at least in endothelial cells [Evans et al., 2001]. In such cells, CD31 confers resistance to cytotoxic stimuli that activate the mitochondrial-dependent cell death pathway. Furthermore, CD31 ligation increased the expression of several protective genes such as the bcl-2 homolog A1, A20, bcl-2 and bcl-xl, and induced Akt phosphorylation and activation [Ferrero et al., 2003]. On the contrary, expression of CD31 truncated molecule inhibited cell proliferation and increased apoptosis, perhaps due to increased association of CD31 with  $\gamma$ -catenin and SHP2 [Ilan et al., 2001].

Here we confirmed the expression of CD31 on some tumor cell lines and its modulation under different culture conditions. Furthermore, we have demonstrated an intracellular pool of CD31 and that engagement of domain 3 of the molecule significantly decreased tumor cell growth by inducing apoptosis.

## MATERIALS AND METHODS

### Cells Lines

The IIB-MEL-LES, IIB-MEL-J, and IIB-MEL-IAN human melanoma cells lines were established from metastatic melanomas

[Guerra et al., 1989; Kairiyama et al., 1995] and the HCT-116 adenocarcinoma cell line was obtained from American Type Culture Collection (ATCC) (Rockville, MD).

#### Tumor Cell Culture

Melanoma cells were routinely grown in Dulbecco's Modified Eagle Medium + Nutrient Mixture F-12 (D-MEM/F-12) (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD) maintained at 37°C in a 5%CO<sub>2</sub> humidified incubator. Adenocarcinoma cells were grown in DMEM supplemented with 10% FBS (Gibco). The monolayer of melanoma cells was detached by treatment with 0.05% EDTA for immunofluorescence studies. In some experiments, monolayers of melanoma cells were allowed to overgrow. Afterwards, the cells that detached spontaneously from the monolayer during the last 16 h of culture were assessed for CD31 expression by immunofluorescence. In order to compare CD31 expression on spontaneously detached and adhered melanoma cells, both group of cells were treated with 0.05% EDTA prior to immunofluorescence staining. In serum starved experiments, melanoma cells were cultured without FBS for 48 h.

#### Human Polymorphonuclear Leukocyte (PMNL) Purification From Normal Donors

Human PMNLs were purified as described previously from ACD-heparin-anticoagulated venous blood of healthy donors [Chuluyan and Issekutz, 1993]. Briefly, leukocyte-rich plasma was collected after red cells were sedimented with 6% dextran-saline (Kabi Pharmacia Uppsala, Sweden). Leukocytes were sedimented and separated by density gradient centrifugation on Percoll gradients. Afterwards, immunofluorescence for CD31 expression was performed.

#### Endothelial Cells

Human umbilical endothelial cells (HUVEC) were isolated and cultured in flasks as described by Jaffe et al. [1973]. Briefly endothelial cells isolated from umbilical cords by collagenase treatment were grown in complete medium composed of RPMI 1640, 2 mM L-glutamine, 2-mercaptoethanol, sodium pyruvate, 20% FBS, 25 µg/ml endothelial cell growth factor, and 22.5 µg/ml heparin in gelatin-coated flasks. The HUVEC were detached by brief treatment with

0.025% trypsin/0.01% EDTA. All the reagents were from Sigma Chemical Co.

#### Monoclonal Antibodies

A number of monoclonal antibodies (mAbs) against human CD31 were generously provided for these studies. These included mAbs PECAM1.1 (IgG2a), PECAM 1.2 (IgG1), and PECAM 1.3 (IgG1) from Dr Peter Newman (Blood Research Institute, Blood Center of Southeastern Wisconsin, Milwaukee), mAb 7E4 (IgG2a) and 1B5 (IgG1) from Dr. Walter Knapp (Institute of Immunology, University of Vienna, Austria), mAb 2B3 (IgG1) from Dr. Ramón Vilella (Servei d'Immunologia, Barcelona, Spain), mAb W6/32 (IgG2a) against HLA-Class I molecules from American Type Culture Collection, ATCC, Rockville, MD. The 5H2 mAb was from a hybridoma that was generated by fusion of the P3U1 myeloma with spleen cells from a BALB/c mouse immunized with purified human PMNL. Standard procedures for fusion and hybridoma culture were used [Kohler and Milstein, 1975]. MAb 5H2 (IgG2a) was used as ascites or cell culture supernatant. This mAb was identified in a screen for inhibition of CD18-independent PMNL transendothelial migration [Issekutz et al., 1995], by reactivity with blood leukocytes, platelets and HUVEC, by immunoprecipitation, and reactivity with recombinant CD31. The screening on constructs containing different domains of CD31 was kindly performed by ELISA in Dr. W. Muller's laboratory (Department of Pathology, Weill Medical College of Cornell University, New York). Monoclonal antibody 5H2 did not bind to constructs containing domain 1 or 1 + 2. It did bind to the constructs containing domain 1–3, with stronger binding to the D1-4 construct. Binding became slightly stronger with each successive domain added. Other antibodies used were: ICAM-1/CD54 (84H10, IgG1, Immunotech, France) and as a control mAb Bi24 (against Lumazine Synthase, IgG2a, kindly provide by Dr. C.A. Fossati) [Goldbaum et al., 1993].

#### Immunofluorescence Staining and Sorting

For immunofluorescence staining, cells were incubated in PBS and 10% heat inactivated goat serum for 30 min at 4°C to block non-specific binding. Subsequently (2–3) × 10<sup>5</sup> cell aliquots were incubated individually with the corresponding mAbs or isotype control mAb at saturating concentration in phosphate buffer

saline (PBS), 1% bovine serum albumin (BSA), 0.1% sodium azide for 45 min at 4°C. Cells were washed twice with PBS-BSA-azide, then treated with phycoerythrin (RPE)-conjugated goat anti-mouse (DAKO, Denmark) immunoglobulin for 45 min at 4°C, washed with PBS-azide and finally resuspended in PBS. The labeled cells were analyzed using a flow cytometer "Cytoron" (Ortho Diagnosis, NJ). In order to measure the total CD31 expression, intracellular plus membrane, the cells were fixed with cold 1% of paraformaldehyde followed by permeabilization with 0.5% saponin (Sigma) in PBS-BSA. After blocking with 10% heat inactivated goat serum for 30 min, unlabeled mAb or isotype control mAb was added (30 min). Cells were washed twice with PBS containing 1% BSA, then treated with phycoerythrin (RPE)-conjugated goat anti-mouse (DAKO, Denmark) immunoglobulin for 30 min; washed with PBS and finally resuspended in PBS. In some experiments, labeled cells were sorted on the basis of their relative expression of CD31 by using a FACStar plus (Becton Dickinson, Mountain View, CA). Two separate populations of melanoma cells, CD31<sup>+</sup> and CD31<sup>-</sup> were obtained. In general, a sort rate of 2,000 cells per second was used. All the staining and sorting was performed using sterile reagents and equipment. The sorted cells were re-analyzed to determine their purity. After sorting, cells were pelleted and the cell number and their viability were determined by trypan blue exclusion.

#### Internalization Assays

The internalization protocol was performed as described previously with modifications [Cefai et al., 1998; Salamone et al., 2001]. In brief, cells were incubated with 10% heat inactivated goat serum for 30 min to block non-specific binding. Then, in order to evaluate the internalization of CD31, the cells were incubated with unconjugated 5H2 mAb for 15–120 min either at 4°C or at 37°C. After washing, the cells were fixed with the A solution of the Fix and Perm kit (Caltag, CA). Aliquots of those cells were further permeabilized with the B solution of the same kit (according to manufacturer's instructions). Afterwards, both fixed and fixed/permeabilized cells were stained for 30 min at room temperature with a FITC-conjugated goat anti-mouse F(ab')<sub>2</sub> immunoglobulin (DAKO, Denmark). Immunofluorescence

was analyzed by confocal microscopy and flow cytometry. Fluorescence was calculated as number of cells with fluorescence intensity exceeding background fluorescence multiplied by the mean fluorescence of CD31-stained cells [Koch et al., 1991].

#### Confocal Microscopy

Color confocal and transmitted light images were acquired with the use of a Zeiss LSM 510 microscope (Carl Zeiss Microscopy, Jena, Germany), with C-apochromat 63×/1.2 W corr. objective. The image size was set to 1,024 pixels with 8 bits pixel depth. The pinhole size was set to 36 μm.

#### Western Blot

For polyacrylamide gel electrophoresis (PAGE) and Western blot, cells were incubated for 1 h in ice with an extraction buffer: TBS 1×, protease inhibitor 1×, CHAPS 1% (Sigma). After centrifugation at 12,000g for 15 min at 4°C, the detergent supernatant was removed and the protein quantified by the Micro BCA<sup>TM</sup> Protein Assay Reagent Kit (Pierce, Rockford, IL). The samples were boiled in sodium dodecyl sulphate-sample buffer containing DTT (0.2M) (Sigma) and analyzed by electrophoresis on a 6% polyacrylamide gel in the presence of SDS. After electrophoresis, gels were transferred to nitrocellulose membrane and subjected to Western blot analysis using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) with a polyclonal Ab to CD31 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:250 dilution and peroxidase rabbit anti-goat secondary Ab (Jackson Immuno Research, Inc., West Grove, PA). Non-immune goat serum was used as negative (antibody) control in these assays.

#### Co-Ligation Assay

This assay was designed to trigger intracellular signaling through CD31 and was performed as described previously [Elias et al., 1998]. In brief, tissue culture plates (96-well flat bottom; Nunc, Rochester, NY) were coated overnight at 4°C with purified 5H2, 7E4, 1B5 or control mAb Bi24 at 20 μg/ml in PBS. After two washings with PBS, mAb-coated wells were incubated with HSA (5 mg/ml) at 37°C for 2 h to block uncoated sites. Melanoma or adenocarcinoma cells were added to each well and incubated for 24 h. Afterwards, all wells

were pulsed with 1  $\mu\text{Ci}$ /well of  $^3\text{H}$ -thymidine (specific activity 5 mCi/mM, Amersham Life Sciences, Arlington Heights, IL). Cellular proliferation of triplicate cultures was determined by quantification  $^3\text{H}$ -thymidine incorporation during the last 18 h of culture.

#### Annexin V Staining of Externalized Phosphatidylserine

Phosphatidylserine exposure was determined by flow cytometric detection of FITC-annexin V staining using the protocol outlined by the manufacturers (Oncogene Research Products, Cambridge, MA) and analyzed with the flow cytometer "Cytoron" (Ortho Diagnosis, NJ).

#### Oligonucleotides

A 21-mer sequence corresponding to the antisense sequence flanking the translation initiation region of the mRNA for CD31 was designed as previously described [Lastres et al., 1994]. The sequence of the phosphorothioate oligonucleotides are as follows with the ATG-initiation codon underlined: antisense CD31: 5'-CCACCTCGGCTGCATCCTGAG-3' and random oligo: 5'-ACCCCAGCTCAGCTCAGCTC-3'.

#### Generation of Transfectants

Tumor cells were transfected with the LipofectAMINE method (LipofectAMINE PLUS Reagent, Gibco, Life Technologies, Grand Island, NY). Briefly, cells were washed with serum-free medium, Opti-MEM (Gibco) containing Lipofectin and oligonucleotides (20  $\mu\text{M}$ ) were added and incubated for 4 h at 37°C. Then, the medium was removed and replaced with the appropriate cell growth medium containing the indicated concentration of oligonucleotides.

#### Statistical Analysis

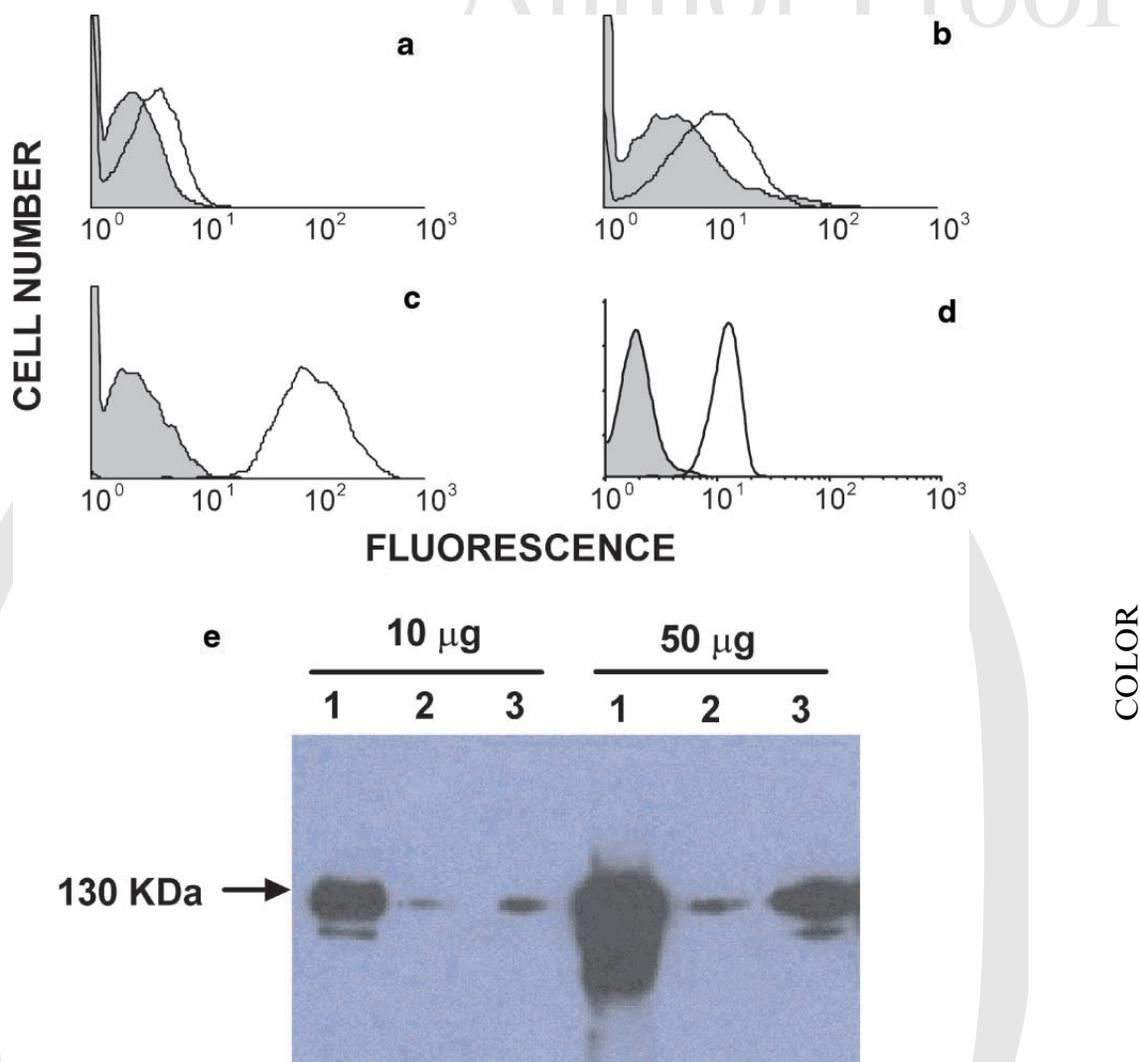
ANOVA were used for statistical analysis of the data, with individual group means compared using post hoc *Student Newman Kules* analysis or Dunnett test as stated.  $P > 0.05$  was considered not significant.

### RESULTS

The potential for tumor cell metastasis is determined in part by adhesion molecules expressed on endothelial and tumor cells. Among the adhesion molecules, CD31 has been shown to be expressed on some rat, mouse, and

human tumor cells lines [Tang et al., 1993]. However, the level of expression seems to vary considerably among cell lines. Furthermore, the role of CD31 in tumor metastasis is not so clear. In order to define the role of CD31 in tumor cell physiology, we first examined the surface expression of CD31 on several cell lines. Figure 1 shows that a melanoma cell line (IIB-MEL-LES) (a) and an adenocarcinoma cell line (HCT-116) (b) expressed low levels of CD31. Low CD31 expression was also observed on other melanoma cell lines, such as IIB-MEL-J and IIB-MEL-IAN (data not shown). This expression was much lower than on endothelial cells (c) or PMNLs (d) (Fig. 1). The low expression of CD31 on cells was also confirmed by performing a Western blot on IIB-MEL-LES, HCT-116, and HUVEC (Fig. 1e). A putative spill over from the lanes containing large amounts of HUVEC CD31 into the adjacent lanes was ruled out by performing the experiments without HUVEC (not shown). It must be noted that the lower molecular weight bands detected with higher protein concentration may be due to protein degradation or a non-reduced/differently glycosylated CD31.

Although the expression of CD31 on tumor cells was consistent within each experiment, we found a variable fluorescence intensity among experiments. This led us to hypothesize that culture conditions such as the state of confluence might influence the expression of CD31. Figure 2a shows that IIB-MEL-LES harvested from confluent cultures (solid line histogram) had lower CD31 expression compared to sparse cultures (broken line histogram). The same was observed for HCT-116 (Fig. 2b). In contrast, HUVEC harvested from sparse cultures (broken line histogram) had lower CD31 expression compared to confluent cultures (solid line histogram, Fig. 2c) as has been previously described [RayChaudhury et al., 2001]. Furthermore, when IIB-MEL-LES with low CD31 expression (harvested from confluent cultures) were seeded again at a lower concentration, they showed CD31 expression similar to cells harvested from sparse cultures (data not shown). This suggested that the expression of CD31 is modulated by cell-cell contact. Indeed, when IIB-MEL-LES were allowed to overgrow, a significant number of cells spontaneously detached from the monolayer. Those detached cells showed higher CD31 surface expression than the cells that remained attached to the



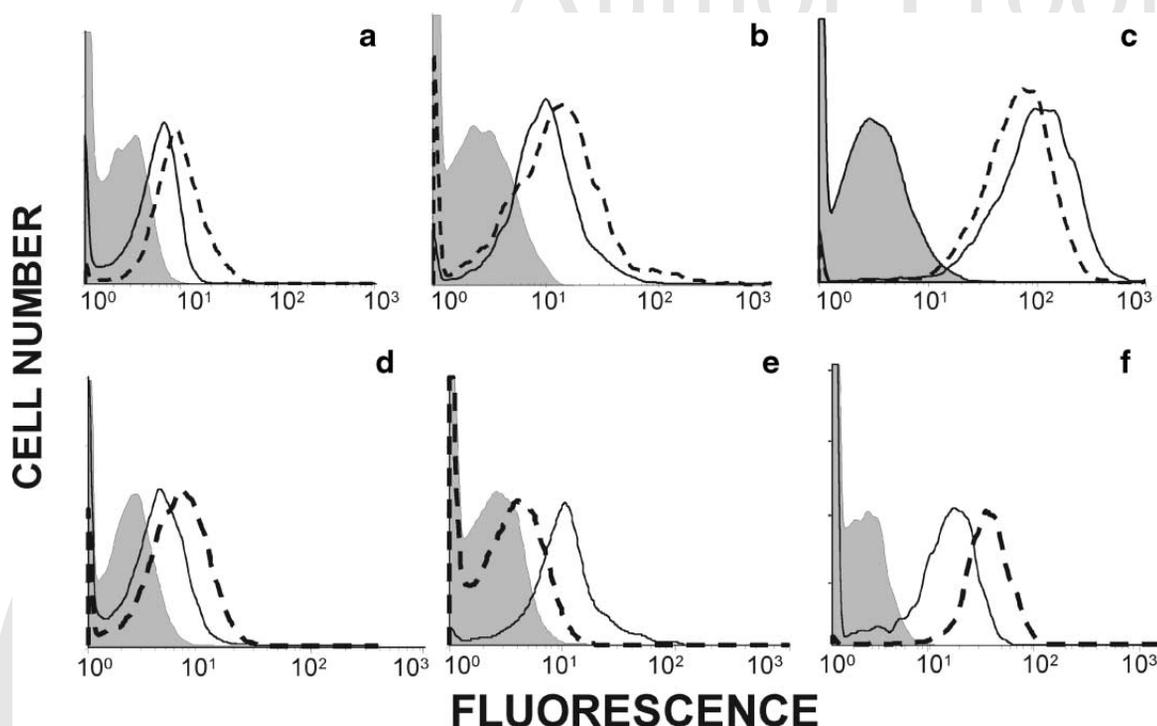
**Fig. 1.** CD31 expression on IIB-MEL-LES (a), HCT-116 (b), HUVEC (c), and PMNLs (d). Cells were stained with mAb 2B3 anti CD31 (unshaded histogram) and analyzed by flow cytometry as described in Materials and Methods. Shaded histogram is isotype control mAb. **e:** Identification of CD31 by Western blot on HUVEC (lane 1), HCT-116 (lane 2), and IIB-MEL-LES (lane 3) with

the C-20 polyclonal antibody (1:250 dilution). Cell lysates with equivalent amounts of protein (10 and 50 µg/lane) were separated on 6% SDS-PAGE. The CD31 band of 130 kDa is indicated by the arrow. Data are representative of three independent experiments.

plastic surface (Fig. 2d). It is important to point out that this expression has been examined only on viable cells ( $78 \pm 3\%$  for adhered cells and  $58 \pm 7\%$  for detached cells), since dead cells were excluded from the analysis using the DNA binding dye, propidium iodide. Furthermore, when spontaneously detached melanoma cells were seeded on culture flasks, they were able to adhere, proliferate, and became confluent. An analysis of the surface expression of CD31 on melanoma cells derived from these confluent cultures showed a downregulation compared to spontaneously detached melanoma cells

(Fig. 2e). In contrast, the expression of other adhesion molecules (CD54 and CD49d) on IIB-MEL-LES did not show changes associated with the state of confluence of the monolayer (data not shown).

The relatively fast changes on the CD31 expression observed on tumor cell lines under different culture conditions may be due to an available putative intracellular pool of CD31. In order to examine the possibility of an intracellular pool of CD31, we performed immunofluorescence studies by fixing and permeabilizing the cells. Figure 2f shows that when cells were fixed



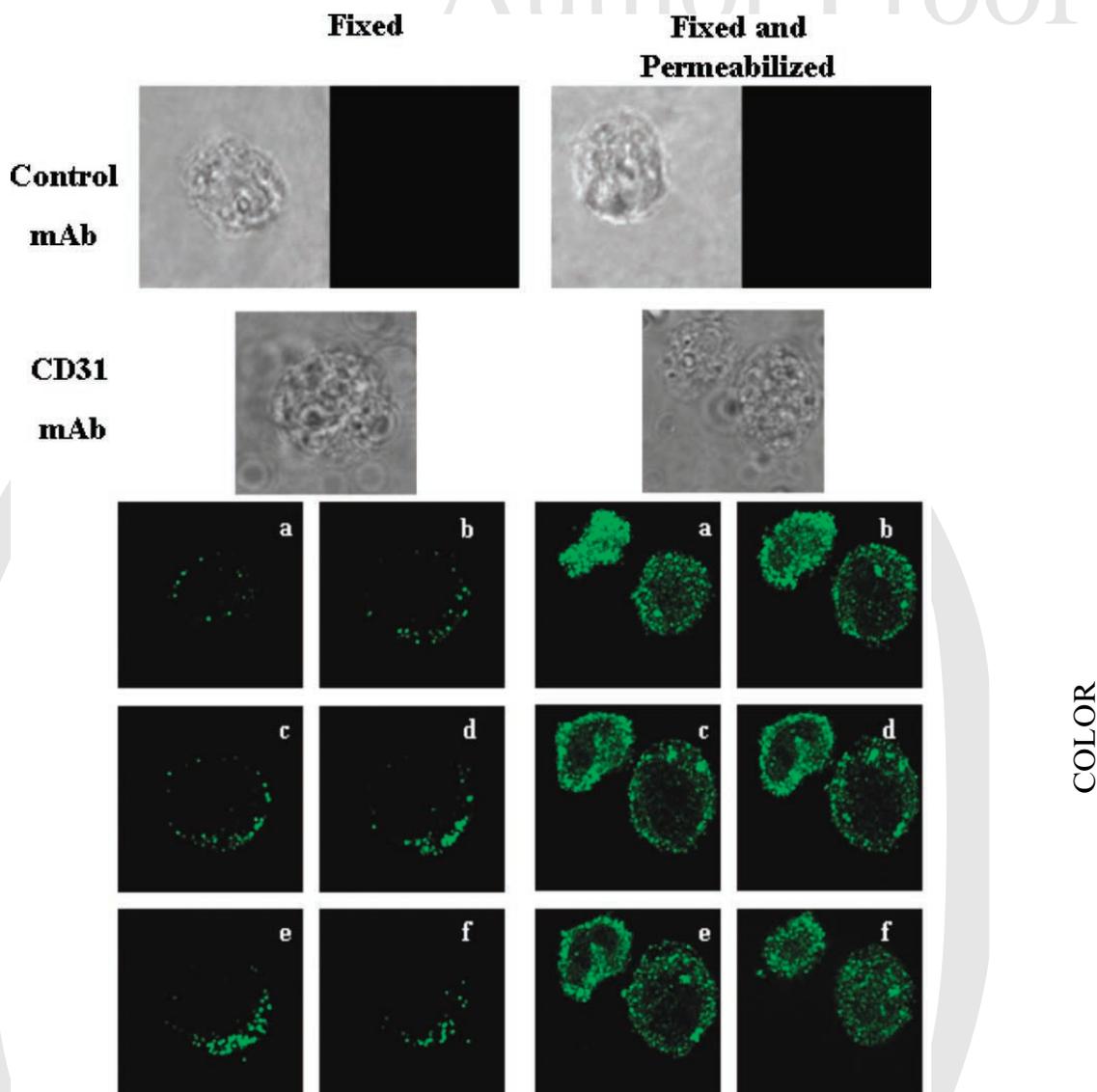
**Fig. 2.** Effect of cell–cell contact on CD31 expression. Melanoma cells IIB-MEL-LES (a), adenocarcinoma cells (b), and HUVEC (c) harvested from confluent (solid line) or sparse (broken line) culture were stained with mAb 5H2. **d:** IIB-MEL-LES spontaneously detached (broken line) from a confluent monolayer (solid line). **e:** Confluent IIB-MEL-LES derived from spontaneously detached melanoma cells (broken line). **f:** Cell surface and total expression of CD31 on IIB-MEL-LES. Cells

were fixed (solid line) or fixed/permeabilized (broken line) with 0.5% saponin and stained with 5H2 mAb as described in Materials and Methods. Isotype mAbs (shaded histogram) were used as negative control for each group in (a), (b), (c), (d), (e), and (f). Experiments were analyzed by flow cytometry after exclusion of dead cells based on propidium iodide staining in (a), (b), (c), (d), and (e). Data are representative of four independent experiments.

and permeabilized (Fig. 2f, broken line), the immunostaining with the mAb to CD31 was much higher than the fixed cells (Fig. 2f, solid line), suggesting the presence of an intracellular pool of CD31. In these experiments, the median fluorescence intensity for the negative control antibody on fixed and fixed/permeabilized cells was the same. These results were definitely confirmed by analyzing the cells by confocal microscopy. Figure 3 shows optical sections of the CD31 distribution on fixed and fixed/permeabilized cells. Whereas on fixed cells 5H2 or 7E4 (not shown) were distributed on some points of the surface of the cells, on fix/permeabilized cells the confocal microscopic analysis showed that 5H2 mAb was distributed in a punctuate globular fashion beneath the cell border (Fig. 3). This staining pattern was stronger above and below the middle plane of the cells and was compatible with the presence of the CD31 associated with a membranous organelle and the cell membrane. It is impor-

tant to mention that these cells were in suspension prior to performing the confocal microscopy. In all the experiments, background threshold levels were set using irrelevant isotype control mouse mAbs. Non-specific binding of mAb 5H2 and 7E4 under fixation and permeabilization was ruled out by performing staining with cells that do not express CD31 such as fibroblasts (data not shown).

The level of CD31 surface expression may be modulated by internalization and exocytosis mechanisms. To determine the presence of an endocytosis mechanism of CD31, we used a protocol previously described [Cefai et al., 1998; Salamone et al., 2001], involving the incubation of the cells with unlabeled mAb (5H2) to CD31 at 37°C and 4°C for 1 h. Afterwards, cells were washed at 4°C to prevent further internalization and fixed or fixed/permeabilized, followed by incubation with goat anti-mouse IgG FITC. In this way, the FITC fluorescence on fixed samples accounted for CD31–mAb complex



**Fig. 3.** Images of CD31 expression on fixed and fixed/permeabilized melanoma cells. After fixing or fixing/permeabilizing, cells were stained with 5H2 mAb or isotype control mAb (IgG2a) and examined by confocal microscopy. Six (a–f) serial representative optical sections through melanoma cells selected from 12 confocal planes from bottom to top. Similar images were obtained in three additional experiments.

that remained on the cell surface after the incubation period (CD31m), while the FITC fluorescence on fixed/permeabilized samples reflected the cell surface-expression (CD31m) plus the internalized mAb–CD31 complex (CD31i). Under these conditions, the CD31m expression on IIB-MEL-LES and HCT-116 was higher at 4°C than at 37°C (Table I), suggesting that at physiologic temperatures the CD31–antiCD31 complex was endocytosed from the cell membrane of tumor cells. On fixed/permeabilized cells there was no difference in the CD31 expression on HCT-116. Furthermore, the

CD31 expression on fixed/permeabilized IIB-MEL-LES was higher at 4°C rather than at 37°C. One possible explanation for this could be the dissociation of the CD31 from the mAb once the complex is internalized or a fast recycling mechanism. To exclude these possibilities, time course experiments were performed on IIB-MEL-LES by using the same protocol. As we show in Figure 4a, the CD31 expression on fixed IIB-MEL-LES at 4°C (CD31m) was higher at any time analyzed. However, the CD31 expression on fixed/permeabilized melanoma cells at 37°C was higher only at 15 and 120 min

**TABLE I. Internalization of CD31 by Melanoma and Adenocarcinoma Cells**

Cells <sup>a</sup>		Incubation temperature		<i>P</i>	N
		37°C	4°C		
CD31m	MEL-LES	239 ± 73 <sup>b</sup>	406 ± 108	0.0305*	5
	HCT-116	363 ± 167	592 ± 178		
CD31m + i	MEL-LES	246 ± 62	425 ± 116	0.0323*	5
	HCT-116	461 ± 167	492 ± 165		

<sup>a</sup>Cells indicated were incubated with mAb 5H2 for 60 min at the indicated temperature prior to fixation CD31m or fixation + permeabilization CD31m + i and followed by secondary FITC Ab staining.

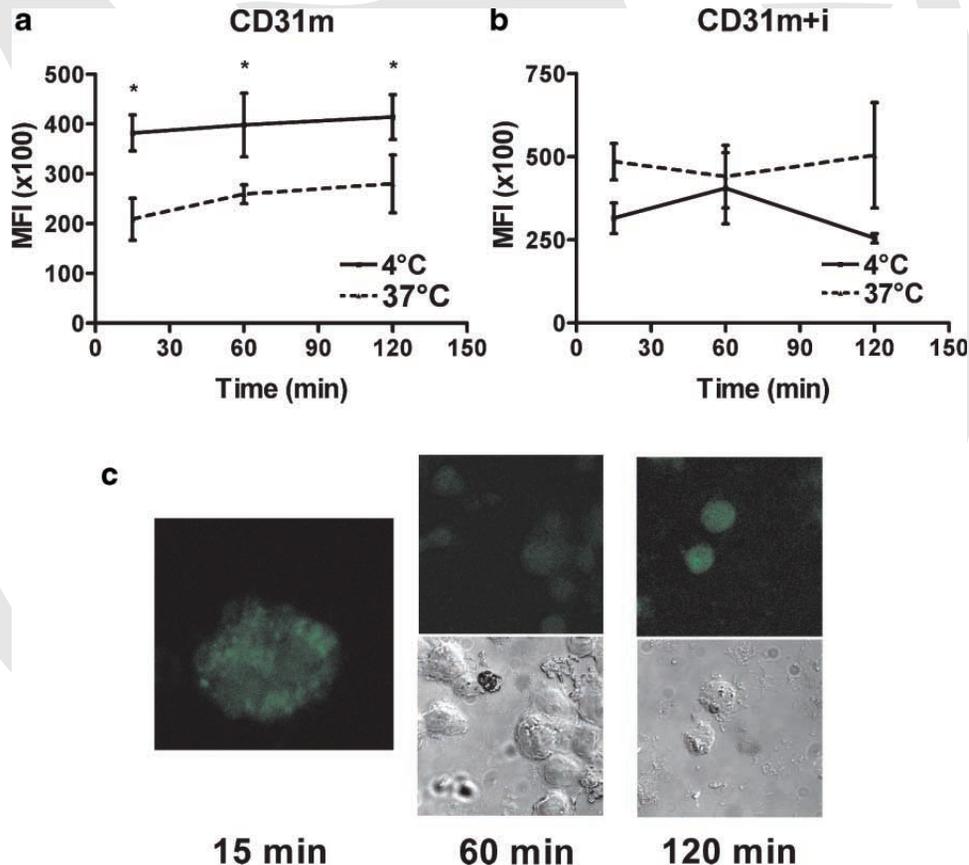
<sup>b</sup>Values are mean fluorescence intensity ± SD of indicated N experiments.

\**P* < 0.05 Student's paired *t*-test.

(Fig. 4b). This was also observed when cells were analyzed by confocal microscopy (Fig. 4c).

Once we established the CD31 expression on some tumor cells, we decided to investigate a putative role of this fast cycling molecule. The

role of CD31 has been extended from vascular cell adhesion to transduce signals that inhibit lymphocyte proliferation [Prager et al., 2001]. Whether CD31 on tumor cells has similar inhibitory effects on proliferation was next



**Fig. 4.** CD31 internalization upon 5H2 mAb binding. CD31/mAb complexes undergo time-dependent internalization. IIB-MEL-LES were incubated at 37°C (broken line) or 4°C (solid line) with 5H2 mAb or isotype control IgG2a (not shown). Aliquots were collected after the indicated time periods and fixed (a) or fixed/permeabilized (b). Afterwards, cells were stained with FITC-conjugated goat anti-mouse F(ab')<sub>2</sub> immunoglobulin and analyzed by flow cytometry. Under these conditions, the FITC fluorescence on fixed cells reflects the cell surface-expression of

CD31 (CD31m). The FITC fluorescence on fixed/permeabilized cells (CD31m + i) reflects the cell surface CD31 that was internalized (CD31i) plus the CD31 that remained on the surface (CD31m). Results are expressed as described in Materials and Methods. Similar results were obtained in four additional experiments. c: Confocal microscopy images of the cells shown in broken lines in panel b; that is fixed/permeabilized cells incubated with the mAb at 37°C.

examined. These experiments were performed by triggering signals through engagement of CD31 with immobilized mAbs against specific domains of CD31. For this, the CD31 on IIB-MEL-LES was ligated with an immobilized CD31 mAb to domain 3 (5H2 or 7E4) or 4–5 (1B5) or an isotype control mAb and then proliferation was assessed. Figure 5 shows that ligation of CD31 with immobilized (a) or soluble (b) 5H2 or 7E4, but not 1B5 or control mAb, significantly decreased the melanoma cell proliferation. The same effect was observed when CD31 on HCT-116 was ligated with immobilized or soluble 5H2 or 7E4 (Fig. 5a,b, respectively). We also assessed the proliferation rate of melanoma cells treated with a specific-CD31 antisense oligonucleotide. Figure 6a shows that CD31 antisense oligonucleotide treated melanoma cells express slightly less surface CD31.

The antisense oligonucleotide treatment was specific for CD31, since other adhesion molecules tested (e.g., ICAM-1/CD54) on melanoma cells were not altered (data not shown). These transfected tumor cells, with lower CD31 expression than control oligonucleotide transfected cells (Fig. 6a), showed a slightly higher proliferation rate than control transfected cells (Fig. 6b). To further assess whether the engagement of CD31 domain 3 results in a decrease in the tumor cell proliferation, we enriched the melanoma CD31<sup>+</sup> population by cell sorting. Figure 7 shows that following cell sorting we recovered a population of CD31<sup>neg</sup> melanoma cells with a 94% of purity (Fig. 7b) and another mixed population (Fig. 7c) of CD31<sup>pos</sup> (43%) and CD31<sup>neg</sup> (57%) from a starting population (a) of CD31<sup>pos</sup> (19%) and CD31<sup>neg</sup> (81%). These populations were cultured on immobilized 5H2 mAb.

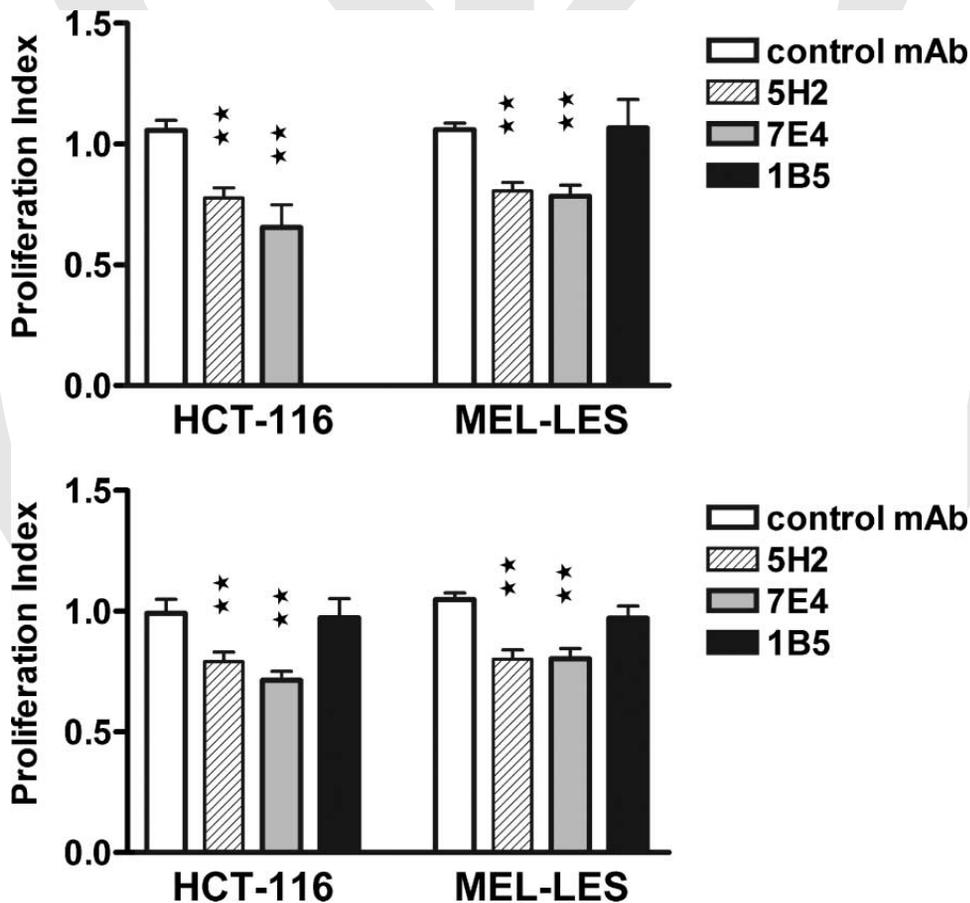
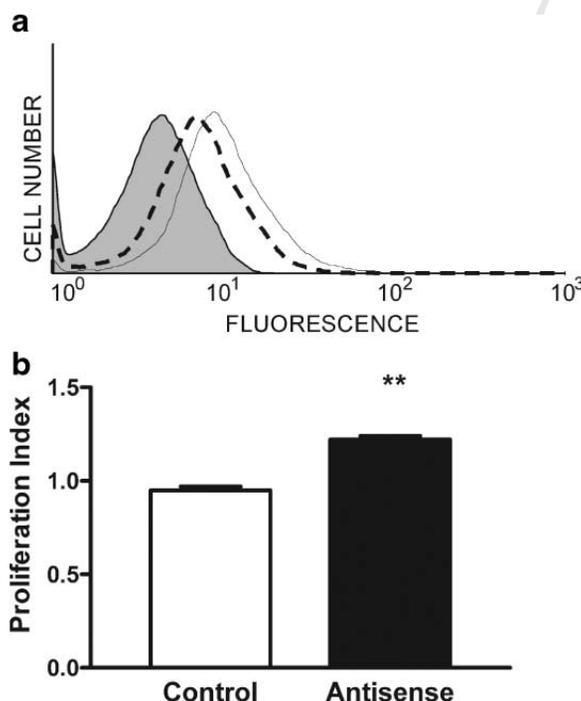


Fig. 5. Effect of CD31 on tumor cell proliferation. The proliferation rate of IIB-MEL-LES and HCT-116 was determined after culture on immobilized (a) or with soluble (b) 5H2, 7E4, and 1B5 mAb. Proliferation was measured as described in Materials and Methods and is expressed as an index relative to cells on control mAb. Data represent the mean  $\pm$  SEM of six independent experiments. \*\* $P < 0.01$ , \* $P < 0.05$  Dunnett multiple comparisons test.



**Fig. 6.** Proliferation of CD31 antisense-oligonucleotide-treated melanoma cells. IIB-MEL-LES cells were incubated with 100 nM of CD31 antisense oligonucleotide (broken line) or Mock oligonucleotide (solid line) as described in Materials and Methods. **a:** Flow cytometry analysis of antisense-oligonucleotide-treated melanoma cells. **b:** Proliferation of antisense or Mock oligonucleotide-treated melanoma cells. Data are expressed as an index relative to lipofectamine treated cells and represent the mean value  $\pm$  SEM of four experiments. \*\* $P < 0.01$ , Student Newman Kules.

Afterwards, proliferation and apoptosis were assessed. After 48 h, the proliferation index of the mixed population corresponding to the cells of Figure 7c showed a very low proliferation index compared to the population of cells from Figure 7b (most of them CD31<sup>neg</sup>) or cells from unsorted melanoma cells ( $0.2 \pm 0.1$  vs  $1.1 \pm 0.03$ ;  $n = 3$ ;  $P < 0.01$ ). To determine whether the inhibition of proliferation was due to apoptosis, we examined the staining of Annexin V on CD31<sup>pos</sup> and CD31<sup>neg</sup> from the mixed population. These experiments were performed with sorted cells (from Fig. 7c) cultured on immobilize 5H2 or 7E4 (data not shown) for 16 h. Figure 7d,e shows that 49% of CD31<sup>pos</sup> cells were apoptotic. However, there was no apoptosis in the CD31<sup>neg</sup> melanoma population.

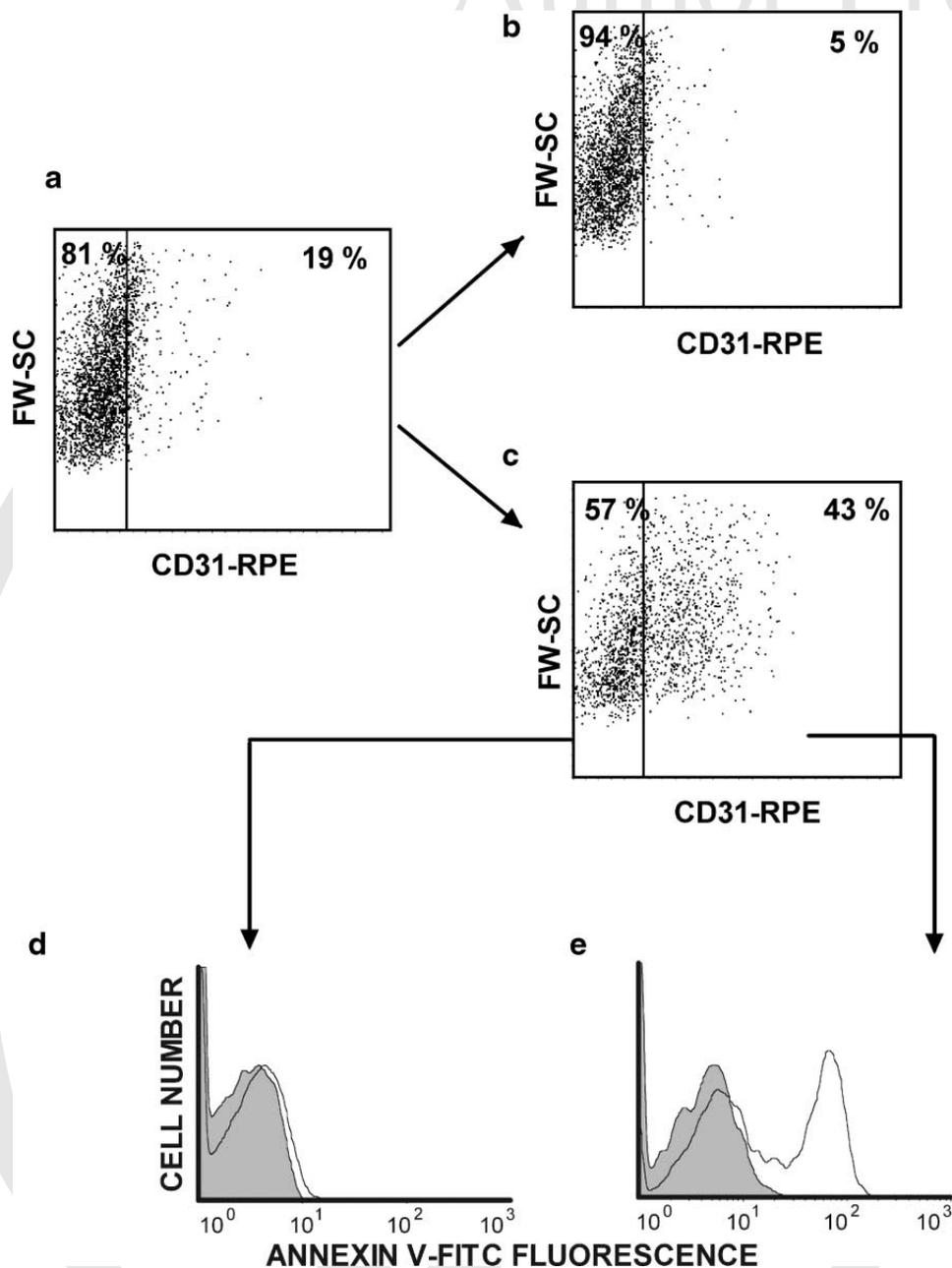
This result was unexpected since it has been described that CD31 protects serum-starved endothelial cells from apoptosis [Evans et al., 2001]. By using a serum-starved melanoma cell

model, we next examined: (i) whether induction or protection from apoptosis depends on the apoptotic model used, and (ii) whether apoptosis induces CD31 expression. For this, once melanoma cells were grown to confluence, the culture medium was replaced with serum-free medium. After 48 h, melanoma were harvested and incubated with CD31 mAb (5H2) or control isotype mAb for an additional 2 h. Finally, apoptosis was evaluated by FACS analysis using annexin V-FITC staining of melanoma cells. Figure 8 shows the FACS dot plots from one representative experiment. In four independent experiments, the percentage of annexin positive melanoma cells in serum-starved cultures was  $30 \pm 1.9\%$ , which was significantly higher than control melanoma cells grown in RPMI-10% FBS ( $18.9 \pm 1.5\%$ ;  $P = 0.03$ ;  $n = 4$ ). An increase in annexin V-FITC positive cells was observed on melanoma cells grown under serum starved conditions after incubation with CD31 mAb compared to control isotype mAb-treated cells ( $30 \pm 1.9\%$  vs.  $47.3 \pm 6.9\%$ ;  $P = 0.045$ ;  $n = 4$ ). However, the CD31 expression on melanoma cells did not change despite the increase in apoptosis observed in serum-starved melanoma cells by signaling through CD31 (Fig. 8).

## DISCUSSION

Adhesion molecules play an important role in cancer metastasis, not only via cell-cell adhesion but also by transduction of signals to the nucleus. We now demonstrate that CD31 expression on tumor cells is involved in a mechanism that may contribute not only to tumor cell adhesion but also signaling which modulates tumor cell proliferation and apoptosis.

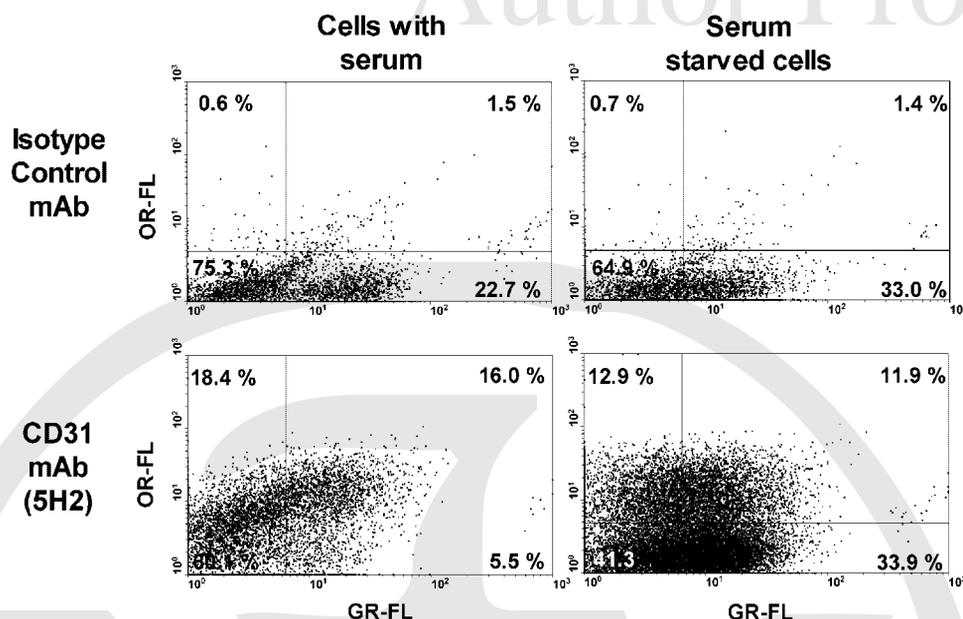
We show that the tumor cell CD31 protein migrates in the range of 130 kDa like that expressed on endothelial cells (Fig. 1). This is quite surprising considering that a marked lineage-specific difference in the molecular mass of CD31 was previously observed [Goldberger et al., 1994]. However, the tumor cell CD31 seems to differ from the CD31 on endothelial cells in a number of aspects. First, the surface expression of CD31 was lower on the melanoma and adenocarcinoma lines studied here than on HUVEC, PMNL or the U937 monocyte-like cells (on line supplemental material). Furthermore, mAbs directed to domain



**Fig. 7.** Cell Sorting and Annexin V staining on melanoma cells. IIB-MEL-LES were stained with 5H2 mAb (a) and enriched by single-cell sorting for CD31<sup>Pos</sup> (c) and CD31<sup>neg</sup> (b) melanoma cells. Cells of Figure 7c were cultured for 16 h. Afterwards CD31<sup>neg</sup> (d) and CD31<sup>Pos</sup> (e) gated cells were analyzed for FITC-annexin V fluorescence. Shaded histogram represents negative control. Similar results were obtained in three additional experiments.

2 (hec7) and 6 (4G6) did not react with CD31 on the melanoma, though they did recognize the molecule on HUVEC and U937 cells (on line supplemental material). This differential reactivity, probably reflects the contribution of carbohydrate residues to the mAb epitope recognition, since carbohydrate moieties are

known to contribute 20%–40% of the molecular mass of CD31 [Newman et al., 1990; Newton et al., 1999]. We cannot rule out differences in the conformation of tumor CD31 due to a mutation that may lead to the loss of the antibody-binding site. Another possible explanation for the differences in the mAb binding



**Fig. 8.** Annexin V and CD31 staining on serum-starved melanoma cells. IIB-MEL-LES were cultured with or without serum medium for 48 h. Thereafter, melanoma cells were harvested and incubated with soluble CD31 mAb (5H2) or control isotype mAb for an additional 2 h. Afterwards, apoptosis was analyzed by FACS analysis and results plotted as a log green (Annexin V) versus log red fluorescence (PE-5H2 mAb). Data are representative of four independent experiments.

may be the generation of different CD31 extracellular domains due to alternative mRNA splicing. Nevertheless, this is less likely since the molecular mass of CD31 present in tumor cells is the same as in HUVEC. However, with HUVEC lower molecular weight bands were observed which were not seen with tumor cells. These low molecular weight bands could be due to protein degradation, a non- or reduced/differently glycosylated CD31 or CD31 cleaved during apoptosis [Ilan et al., 2001]. Alternative splicing that generates multiple CD31 isoforms has been described for the cytoplasmic tail [Kirschbaum et al., 1994] but not yet for the extracellular domains.

Second, the regulation of the CD31 expression on tumor cells differs from endothelial cells, since at subconfluence (sparse growth) endothelial cells downregulate CD31 expression [Ray-Chaudhury et al., 2001]. In contrast, at subconfluence the tumor cells examined here expressed more CD31 than at confluence. It is possible that the expression of CD31 on tumor cells is downmodulated by cell-cell contact. Perhaps, CD31 is internalized when cells come in contact, thus avoiding a proliferation inhibitory signal. The CD31 downregulation may possibly release the cells from the monolayer and in vivo may facilitate metastasis from the

primary tumor. This is supported by the finding that tumor cells which lost cell-cell contacts and spontaneously detached, expressed more surface CD31. These CD31<sup>POS</sup> cells may be more efficient in the adhesion to endothelial cells and in forming metastases, since adhesion of melanoma cells and adenocarcinoma cells are partially mediated by CD31 [Tang et al., 1993]. In addition, the adhesion to HUVEC of tumor cells derived from subconfluent (sparse) monolayers that expressed more CD31 (Fig. 3) was higher than with cells derived from confluent monolayers (on line supplemental material). Furthermore, the adhesion of CD31-antisense oligonucleotide transfected melanoma cells to HUVEC was lower than mock transfected cells (on line supplemental material). Interestingly, the endothelial adhesion role of CD31 on melanoma cells appears to be different from leukocytes such as monocytes, since domain 1, 3-5 appears to mediate melanoma adhesion, while blockade of these domains had no effect on monocyte adhesion (on line supplemental material). Although, we do not exactly know what happens in real tumors, there are some reports showing a weak cytoplasmic staining in tumors [Miettinen et al., 1994; De Young et al., 1998]. Indeed, the confocal studies and the CD31 expression on fixed and permeabilized cells,

support the presence of an intracellular pool of CD31 on some tumor cells, such as the one described in platelets [Cramer et al., 1994] and endothelial cells [Mamdouh et al., 2003]. This intracellular pool may be mobilized under certain conditions, perhaps facilitating metastasis. Furthermore, the mechanisms of internalization described herein may explain the difficulties in detecting CD31 membrane expression in tissue fragments [De Young et al., 1998]. CD31 expression on endothelial cells is constitutive but there are a few factors described that can modulate this expression. Cytokines can modulate CD31 expression on U937 [Lastres et al., 1994] and endothelial cells. For example,  $\text{TNF}\alpha$  and  $\text{IFN}\gamma$  induce a redistribution of the molecule away from intercellular junctions [Romer et al., 1995], whereas the combination of both cytokines caused the disappearance of CD31 from the membrane [Rival et al., 1996]. This effect was related to the internalization and degradation of pre-existent CD31 and the inhibition of its synthesis [Rival et al., 1996]. On tumor cells, similar mechanisms may be operative.

Mechanisms of endocytosis and recycling can modulate the expression levels of several molecules [Gordon and Lloyd, 1994]. Endocytosed molecules first appear in early endosomes, from where they can be recycled back to the cell surface and/or forwarded for proteolytic degradation in late endosomes and lysosomes [Sandoval and Bakke, 1994]. This endocytic pathway intersects with the secretory pathway, allowing the delivery of newly synthesized glycoproteins to late endosomes/lysosomes [Sandoval and Bakke, 1994; Gagescu et al., 2000]. For some receptors the process appears constitutive, whereas for others it is ligand-induced (e.g., CD5) [Shawler et al., 1988]. Among adhesion molecules the expression of E-cadherin seems to be regulated by endocytosis [Le et al., 1999].

It is known that the traffic of internalized membrane proteins is determined by a variety of tyrosine and leucine-based sorting motifs present in the cytoplasmic tails of proteins [Sandoval and Bakke, 1994; Ohno et al., 1995; Marks et al., 1996]. Four types of endocytic sorting motifs can be found in the cytoplasmic tail of CD31 (i.e.,  $\text{L}^{644}\text{L}^{645}$ ;  $\text{N}^{660}\text{SHY}^{663}$ ;  $\text{Y}^{689}\text{TEV}^{692}$ ;  $\text{Y}^{713}\text{SEV}^{716}$ ), supporting the concept of an endocytic and recycling mechanism for CD31. On tumor cells, the internalization of

the CD31 is another regulatory mechanism for the surface CD31 expression that seems to take place. This is supported by the experiments that showed a downregulation of the surface molecule when the cells were preincubated with the unlabeled mAb at 37°C compared to 4°C. Several intracellular routes have been described for many membrane molecules after endocytosis; for instance: (i) rapid recycling back to the cell membrane via early endosomes [Killisch et al., 1992]; (ii) slower recycling via a pathway involving the *trans*-Golgi complex [Clayton et al., 1992]; and (iii) a route to lysosomes and degradation after internalization [Stoscheck and Carpenter, 1984]. Since the rate of the 5H2 or 7E4 mAb uptake was not linear, we can assume that CD31 could be included in the latter group. Furthermore, the rapid saturation of the intracellular compartment by the CD31–antiCD31 mAb complex, followed by a decay (60 min) after its internalization, also suggests that a proteolytic compartment could be involved. Although direct evidence for the predominant route taken by CD31 is not yet available, our preliminary data (not shown) indicates that the principal pathway involves recycling of CD31 via a cytoskeleton dependent and brefeldine-A sensitive route (data not shown). However, it is important to mention that two papers have described the internalization of CD31 on endothelial cells [Mamdouh et al., 2003; Muro et al., 2003]. The first one identified a new membrane compartment at the intercellular borders of endothelial cells that is distinct from caveola and endosomes [Mamdouh et al., 2003]. The second report indicated the existence of a novel endocytic pathway in the internalization of clustered ICAM-1 and CD31 on endothelial cells [Muro et al., 2003]. Based on the staining pattern found on melanoma cells, we can assume that both the novel endocytic pathway and the membrane compartment described for endothelial cells could be operative in melanoma cells. Thus the specific intracellular pathways involved in the CD31 exo and endocytosis and subcellular localization on tumor cells warrants further studies.

CD31 belongs to the newly defined Ig-ITIM superfamily of receptors, which are characterized by the presence of intracytoplasmic ITIM that recruit and activate protein-tyrosine phosphatases [Newman, 1999]. Interestingly, those ITIM motifs allocate the endocytic-sorting

motif described above: V<sup>688</sup>QYTEV<sup>693</sup>; T<sup>711</sup>VYSEV<sup>716</sup>. The relevance of CD31 and its inhibitory motifs in tumor cells became clear from the co-ligation experiments, since the engagement of CD31 on cells inhibited their proliferation only on CD31<sup>pos</sup> but not on CD31<sup>neg</sup> melanoma cells. Based on the serum starved culture experiments, we can assume that apoptosis does not increase CD31 expression. On the contrary, it is the signaling through CD31 that triggers apoptosis on some tumor cells. A modulatory effect of anti-CD31 antibody engagement on CD31 has been also described for lymphocytes [Elias et al., 1998; Newman, 1999; Newton-Nash and Newman, 1999], monocytes, neutrophils [Berman and Muller, 1995], NK [Berman et al., 1996], and endothelial cells [Nelissen et al., 2002] which leads to protection from starvation-induced apoptosis [Ferrero et al., 2003; Gao et al., 2003]. However, it has been shown that the stable transfection of a truncated CD31 gene construct in colon carcinoma cells, resulted in decreased cell proliferation by increasing programmed cell death [Ilan et al., 2001]. Our results reinforce this finding and the concept that the CD31 function on tumor cells seems to differ from that on normal vascular cells, at least in the survival effects and in the modulation of the surface expression.

Although we used tumor cell lines, which may be distinct from their source tissue, and an in vitro approach to unravel the role of CD31 on tumor cells, we consider that these findings are likely relevant to cancer. We conclude that it is possible that CD31 may be mediating tumor cell adhesion and/or be involved in the cell cycle proliferation in a phenotypic subpopulation of cells, that is, at a particular phase in the cell cycle or differentiation stage. Likely the balance in the level of expression of CD31 and other adhesion molecules on tumor cells may vary during the life of that cell and this could result in changes in the proliferation rate. These findings warrant further assessment of tumor cell CD31 in situ and might be contribute to developing anti-metastatic therapy that would affect the proliferation rate of certain types of tumor cells.

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