Localization of integrin αvß3 and vascular endothelial growth factor receptor-2 (KDR/Flk-1) in cutaneous and oral melanomas of dog

N.G. Rawlings[1], E. Simko[2], T. Bebchuk[3], S.J. Caldwell[1] and B. Singh[1]

Departments of [1]Veterinary Biomedical Sciences, [2]Veterinary Pathology and

[3]Small Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada

Summary. Melanomas are common neoplasms of dogs and arise from pigment-producing cells called melanocytes or melanoblasts. Melanomas of skin are often easily cured by surgical excision, but those of oral mucosa are aggressive, metastasize to the regional lymph nodes and lungs, and respond poorly to conventional therapy. Tumor growth is sustained by proliferation of microvessels via a process called angiogenesis. Integrin αvß3 is expressed in proliferating but not in quiescent microvessels suggesting a role in angiogenesis. Vascular endothelial growth factor (VEGF) manifests its mitogenic and angiogenic effects mainly via VEGF receptor-2 (VEGFR-2/Flk-1). We conducted this immunocytochemical study to investigate the expression of integrin αvß3 and VEGFR-2 in archival and fresh samples from cases of canine melanomas. Results show that integrin αvß3 was expressed in 72% and 88% of cutaneous and oral melanomas, respectively, and the expression was restricted to and immediately around the melanocytes and endothelial cells. VEGFR-2 staining of selected cases of melanoma revealed that its expression overlapped with the αvβ3 integrin. Dual immuno-gold electron microscopy confirmed co-localization of integrin αvß3 and VEGFR-2 in melanocytes and endothelial cells. These data demonstrate expression and co-localization of integrin αvß3 and VEGFR-2 in cutaneous and oral melanomas of dogs.

Key words: Integrin αvß3, VEGFR-2/flk-1, Melanoma, Dog, Immunocytochemistry

Offprint requests to: Baljit Singh, BVSc&AH, PhD, Department of Veterinary Biomedical Sciences, 52 Campus Drive, University of Saskatchewan, Saskatoon, SK, S7N 5B4, Canada. Fax: 306.966.7405. e-mail: baljit.singh@usask.ca

Introduction

Melanomas arise from melanocytes and comprise up to 7% of all canine neoplasms (Modiano et al., 1999). These tumors can afflict any breed of dogs but are more prevalent in Schnauzers, Doberman Pinschers, Scottish Terriers, and Golden Retrievers (Modiano et al., 1999). The majority of oral melanomas are highly aggressive, metastasize to the regional lymph nodes and lungs, and respond poorly to conventional therapies (Whartenby et al., 1995; Dow et al., 1998; Modiano et al., 1999). Cutaneous melanomas have a better prognosis following surgical intervention. The cellular and molecular mechanisms that induce diversity in biological behavior of cutaneous and oral melanomas are not precisely understood (Modiano et al., 1999). Since this diversity appears to influence responses of canine melanomas to therapy, it is critical to characterize the molecular mechanisms that influence this behavioral diversity.

Tumors require extensive vascular networks for their growth and survival (Friedlander et al., 1995; Whartenby et al., 1995). Neoplastic invasion into surrounding healthy tissues is controlled in part by the proliferation of existing vasculature through a process called angiogenesis. Angiogenesis is a complex process believed to be regulated by the interplay of various factors including adhesion molecules such as integrins and extra-cellular matrix components such as vitronectin (Lymboussaki et al., 1998). Integrins are heterodimers composed of α and ß subunits that mediate cell adhesion and initiate cell signaling (Gahmberg et al., 1998). The αvβ3 integrin, also known as a vitronectin receptor, is constitutively expressed in epithelia of various organs but not in resting microvessels except those of rat lungs (Brooks et al., 1994a; Singh et al., 2000, 2001).

The expression of integrin αvβ3 is strikingly increased in the microvasculature of embryos, wounds, rheumatoid arthritis and melanoma tumors in humans (Byzova et al., 1998; Storgard et al., 1999). The αvβ3 integrin facilitates attachment of endothelial cells to

components of extra-cellular matrix such as vitronectin and promotes their survival by stimulation of anti-apoptotic mechanisms including inhibition of p53 and increased levels of bcl-2 protein (Brooks et al., 1994b). The integrin also enhances endothelial cell migration and invasiveness via interaction with the matrix components and co-localization with matrix metalloproteinase-2 (Brooks et al., 1996). There is evidence that integrin αvβ3 stimulates transition from the radial phase of growth to the invasive and malignant vertical phase in human melanomas (Natali et al., 1997; Clezardin et al., 1998; Hus et al., 1998). Furthermore, inhibition of integrin αvβ3 by a monoclonal antibody or integrin-specific RGD peptides disrupts the microvascular network and causes regression of human melanomas (Brooks et al., 1994b; Storgard et al., 1999).

Vascular endothelial growth factor (VEGF) increases microvascular permeability and is also an endothelial cell survival factor although it may not be critical for the normal development of blood vessels in the embryo (Shapiro, 2000; Ferrara, 2001). VEGF binds to two tyrosine kinase receptors expressed on endothelial cells: VEGF receptor-1 (VEGFR-1; Flt-1) and VEGFR-2 (KDR/Flk-1). VEGF ligation of VEGFR-2 activates cell signaling via tyrosine phosphorylation and increases mitogenicity and angiogenicity of endothelial cells (Byzova et al., 2000). There is evidence that VEGFR-2 is expressed in human melanoma cell lines and that anti-VEGFR-2 antibody disrupts tumor angiogenesis and inhibits growth of many tumors including human melanoma (Prewett et al., 1999).

Cutaneous and oral melanomas of dogs differ in their aggressiveness and response to surgical interventions. The mechanisms of this diversity are not fully understood. There are no data on the expression of integrin αvβ3 and VEGFR-2, the key regulators of angiogenesis and malignant transformation, in cutaneous and oral melanomas of dogs. Therefore, we conducted an immunocytochemical study to examine the expression of the αvβ3 integrin and VEGFR-2 in cutaneous and oral melanomas in the dog.

Materials and methods

Melanoma samples

Canine melanoma samples (n=75) were obtained from the archives of the Department of Veterinary Pathology, University of Saskatchewan. These tissues were collected between 1989 and 1999 from male and female dogs of various breeds. Histological sections from these tumors were reviewed and divided into two main categories of melanoma: cutaneous (n=42) and oral (n=33). Three fresh biopsies obtained from clinical cases of melanoma brought to the Veterinary Teaching Hospital of University of Saskatchewan were fixed in 4% paraformaldehyde for 16 hours at 4 °C and embedded in paraffin for light microscopy. For electron microscopy, the biopsies were processed as reported previously (Singh et al., 2001). Briefly, the tissues were

fixed for 3 hours in 0.1% glutaraldehyde plus 2.0% paraformaldehyde in 0.1M sodium cacodylate buffer and embedded in LR White Resin (London Resin Company).

Integrin αvß3 and VEGFR-2 immunocytochemistry

Sections (5-7 μm) were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol followed by incubation with 5% hydrogen peroxide in methanol for 20 minutes to block the endogenous peroxidase. After a rinse in water, the antigenic sites were exposed by incubation in 2mg/ml pepsin in 0.01 N HCl for 75 minutes. The digestive action of pepsin was terminated by a wash in phosphate-buffered saline (1x PBS, pH 7.3). To prevent non-specific binding, the sections were blocked with 1% BSA in PBS. The consecutive sections were incubated with an anti-αvβ3 monoclonal antibody (1:50, MAB1976/LM609, Chemicon Inc. Temecula, USA) or anti-VEGFR-2 (1:10; Flk-1; Santa Cruz Biotech Co., USA) antibody for 60 minutes followed by a goat anti-mouse IgG secondary antibody conjugated with horseradish peroxidase (1:100; DAKO Corp. Carpinteria, USA) for 30 minutes. The color was developed for 10 minutes with a commercial kit (Vector Laboratories, Canada) and then rinsed in running water for 5 minutes. The controls consisted of sections incubated with only secondary antibody or with primary anti-von Willebrand Factor antibody (vWF 1:150, A 0082, DAKO Corp., Carpinteria, USA).

Immuno-electron microscopy

One μm thick sections stained with toluidine blue were examined to select desirable areas for ultra-microtomy. Thin sections (90 nm) were collected on meshed nickel grids blocked with albumin and exposed to anti-αvβ3 integrin antibody (1:50) or anti-VEGFR-2 (Flk-1; 1:10) for 60 minutes. The grids were washed in tris-buffered saline and then incubated with gold-conjugated anti-mouse secondary antibodies (1:40) for 60 minutes. Dual immunogold labeling was performed to confirm if integrin αvβ3 and VEGFR-2 are present in the same cells. One side of each section was first stained with the αvβ3 integrin antibody followed by incubation with secondary antibody conjugated to 12 nm gold particles. Then, the other side was exposed to anti-VEGFR-2 antibody and 15 nm gold particle-conjugated secondary antibody. The sections were stained with 2% aqueous uranyl acetate with triton-X and lead citrate followed by examination in an electron microscope at 60kV.

Results

Light microscopic immunocytochemistry

Integrin αvß3

The sections from cutaneous (Fig. 1A) and oral (data not shown) melanomas that were not exposed to a
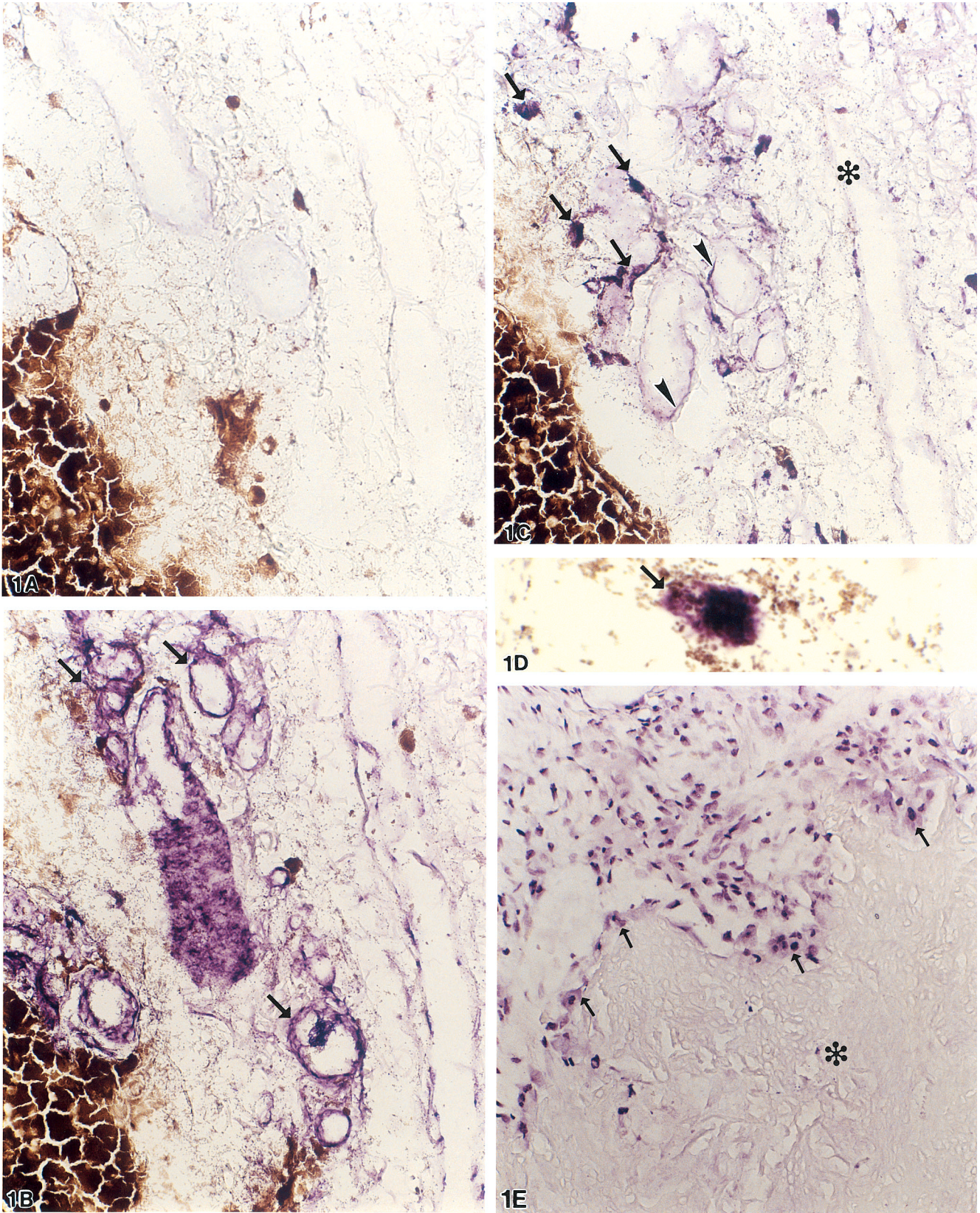
Fig. 1. Consecutive sections **(A-C)** from a case of cutaneous melanoma: Negative control **(A)** shows no staining while vWF antibody **(B)** revealed blood vessels (single arrows). Integrin αvß3 antibody **(C)** stained neoplastic melanocytes (single arrows) and blood vessels (arrowheads) but not the tissue areas away from melanocytes (asterisks). High magnification view **(D)** of a melanocyte (arrow) shows reaction for integrin αvß3. **E.** taken from a case of oral melanoma shows integrin αvß3 staining in neoplastic melanocytes (single arrows) and demarcates them from the adjacent areas (asterisks). A-C, E, x 100; D, x 400

2A

2B
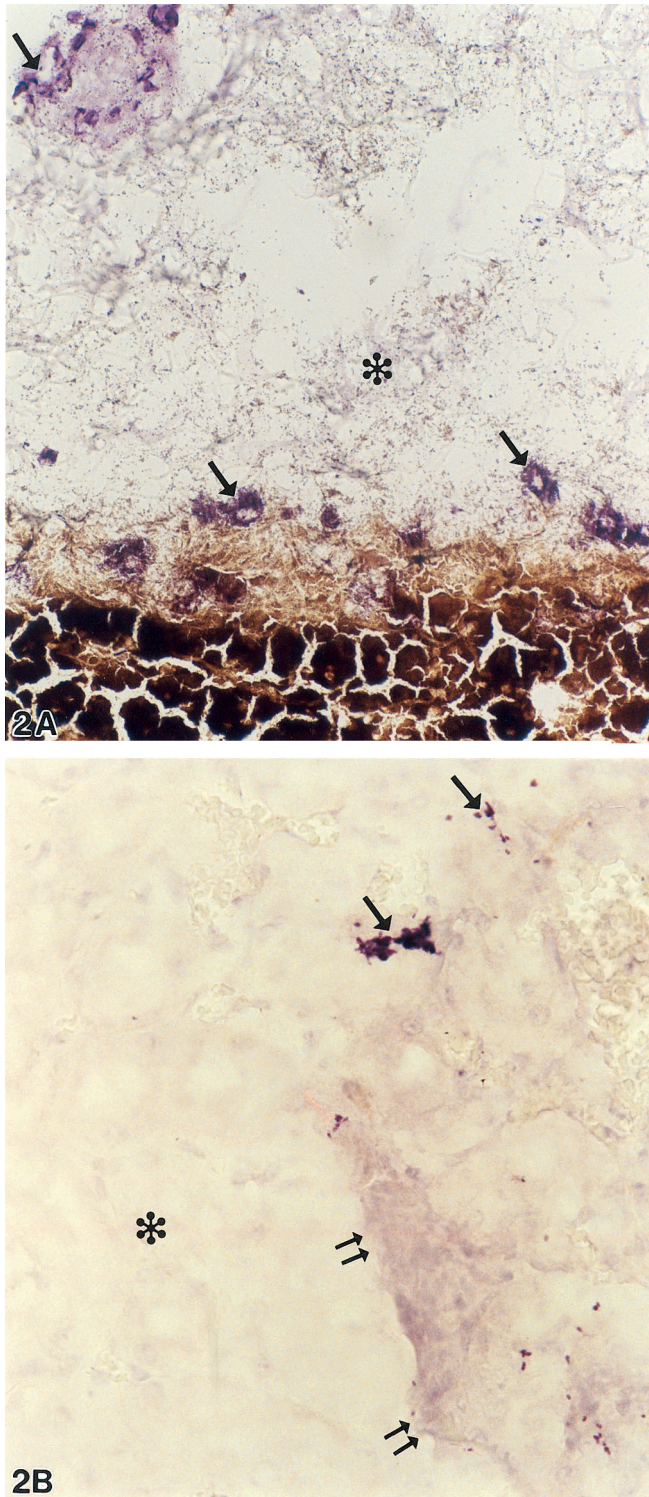
Fig. 2. A section taken from a case of cutaneous melanoma **(A)** shows VEGFR-2 staining in neoplastic melanocytes (single arrows) and their immediate vicinity but not in surrounding areas (asterisks). **B.** Taken from a section of oral melanoma shows VEGFR-2 staining in neoplastic melanocytes (single arrows) and demarcation (double arrows) from the surrounding areas (asterisks). x 100

primary antibody lacked any staining while incubation with anti-vWF antibody delineated blood vessels (Fig. 1B). Integrin αvß3 was localized in cutaneous (Fig. 1C) and oral melanomas (Fig. 1E). The integrin staining was primarily concentrated in and around the tumor cells (Fig. 1D) as well as microvascular endothelial cells but not in the surrounding normal regions of the tissues (Fig. 1C-E). Moreover, the staining was observed primarily in the melanocytes at the margins (Fig. 1C,E) of the main mass of the melanoma and created a clear demarcation from the apparently healthy tissues. Though subjective, the intensity of integrin αvβ3 appeared to be higher in melanomas of oral mucosa compared to those of the skin. We found that 72% (30 out of 42 cases) of cutaneous melanomas and 88% (29 out of 33 cases) of oral melanomas were positive for integrin αvß3.
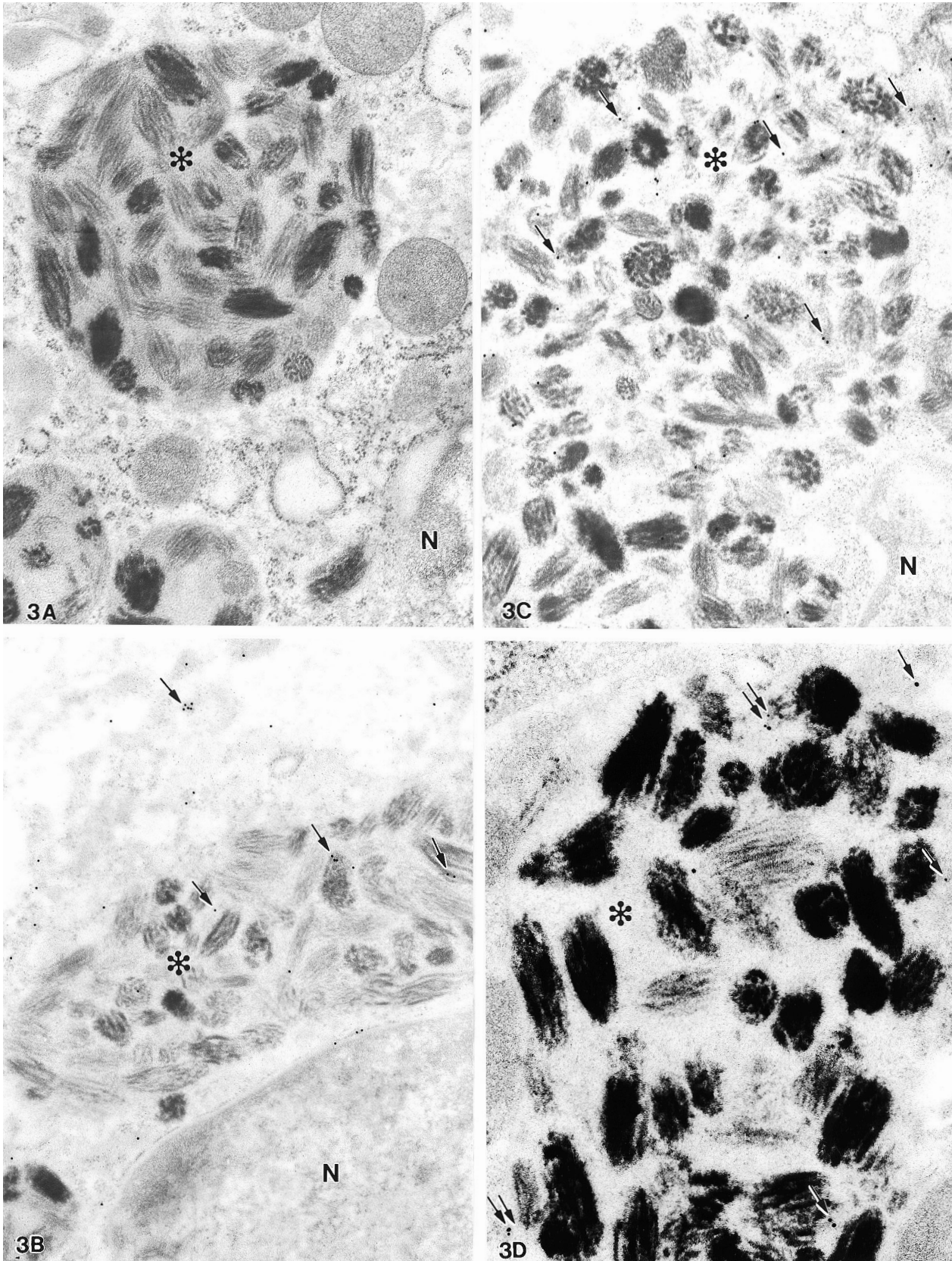
VEGFR-2

We performed staining for VEGFR-2 on selected cases only that were either positive (n=32) or negative (n=4) for the integrin. The pattern of VEGFR-2 reactivity was similar to that of the integrin and was observed in melanocytes and endothelial cells of cutaneous melanomas (Fig. 2A). In oral melanomas, VEGFR-2 antibody stained melanocytes and areas surrounding them (Fig. 2B). Similar to the integrin staining, the VEGFR-2 was also absent in tissue areas away from neoplastic cells in cutaneous and oral melanomas (Fig. 2A-B).

Immuno-electron microscopy for integrin αvß3 and VEGFR-2

We performed immuno-electron microscopy on two biopsies of oral melanomas to confirm and further extend the immuno-histological data. The sections exposed only to the secondary antibodies lacked any labeling with gold particles (Fig. 3A). Melanosomes and endothelial cells in the tumors stained with an anti-integrin αvß3 antibody (Fig. 3B). The sections from the same tissue block probed with an anti-VEGFR-2 antibody also showed prominent labeling in melanosomes of neoplastic melanocytes (Fig. 3C). The staining pattern of integrinαvß3 and VEGFR-2 suggested co-localization of both the proteins in melanocytes and endothelial cells. This suspicion was confirmed by dual immuno-gold labeling on the same sections for the αvß3 integrin and VEGFR-2 and it showed staining for both the markers in the same melanocytes (Fig. 3D). These 12 nm and 15 nm gold particles were present in close proximity as well as individually in different areas of the melanocytes.

Discussion

We report the expression of the αvß3 integrin in 88% of oral and 72% of cutaneous melanomas compared to lack of staining in normal tissues. The degree of

Fig. 3. Sections stained without primary antibody **(A)** lack gold labeling while those stained with integrin αvß3 **(B)** and VEGFR-2 **(C)** antibodies show labeling (single arrows) in melanosomes (asterisks) and cytosol of melanocytes. Dual-labeling **(D)** for the αvß3 integrin (12 nm gold particles) and VEGFR-2 (15 nm gold particles) shows co-localization of both the proteins (double arrows) while single arrows indicate individual gold particles. N: nucleus. x 37,500

expression of the integrin αvβ3 in cutaneous and oral melanomas is similar to that previously reported by Natali and colleagues in malignant cutaneous melanomas of humans (Natali et al., 1997). Although oral melanomas are more aggressive than cutaneous melanomas, no differences were detected in the expression of integrin αvβ3 between the two.

The αvß3 integrin was tightly restricted to and around melanocytes in canine melanomas and was absent in normal tissues. These observations are similar to the previously reported lack of expression of integrin αvβ3 in normal skin and other tissues, and resting microvessels (Natali et al., 1997; Eliceiri and Cheresh, 1999; Singh et al., 2001). Since the integrin regulates microvascular development, its expression is robust in proliferating microvessels in embryo, tumors and wound repair (Brooks et al., 1994a; Friedlander et al., 1995; Clark et al., 1996; Peticlerc et al., 1999; Storgard et al., 1999). Integrin αvß3 promotes microvessel proliferation by inhibiting activity of p53 tumor suppressor protein and increasing levels of anti-apoptotic proteins such as bcl-2 (Strombald et al., 1996; Seftor, 1998; Seftor et al., 1999). Recently, Modiano and colleagues reported a lack of p53 translocation to nuclear compartments in cells in canine melanomas (Koenig et al., 2002). It is possible that integrin αvß3 may participate in suppression of p53 to promote angiogenesis and tumorigenicity of melanocytes and increase survival of canine melanomas.

These are the first data on the VEGFR-2 expression in cutaneous and oral melanomas of dogs. Immunocytochemical data show VEGFR-2 in and around the neoplastic melanocytes and endothelial cells. Similar to the integrin αvβ3 expression, the VEGFR-2 was also not observed in tissue areas away from the melanocytes. These results are consistent with previous observations on restricted expression of VEGFR-2 in human melanoma cell lines and dividing endothelial cells compared to normal endothelial cells and melanocytes (Liu et al., 1995; Graeven et al., 1999; Heidenreich et al., 2000; Ferrara, 2001). The VEGF/VEGFR system promotes angiogenesis since VEGF$^{-/-}$/VEGFR$^{-/-}$ mice lack vascular development and show increased early embryonic lethality (Ferrara, 2001). The mitogenic and angiogenic effects of VEGF are transduced primarily via VEGFR-2 that facilitates tyrosine phosphorylation and cell signaling (Ferrara, 2001). VEGFR-2 seems to be a critical factor in melanomas since immunization against VEGFR-2 inhibited tumor angiogenesis and metastasis following challenge with B16 melanoma cells (Li et al., 2002). Recently, a correlation was demonstrated between expression of VEGFR-2 and Ki-67 in thicker human melanomas indicating its role in angiogenesis and tumor growth; Ki-67 is accepted as a reliable prognostic marker of canine melanomas (Roels et al., 1999; Koenig et al., 2001; Straume and Akslen, 2001; Koenig et al., 2002). There may be a putative interaction between VEGFR-2 and Ki-67 to influence growth of canine

melanomas. Therefore, further studies are needed to establish a precise role of VEGFR-2 in canine melanomas with an intent to exploit it's potential as a therapeutic target.

A novel observation is the expression of integrin αvβ3 and VEGFR-2 in melanocytes and endothelial cells. This was confirmed with dual immuno-electron microscopy which is a powerful technique for spatial and subcellular localization of two different molecules in the same tissue section. This is the first direct evidence on the co-localization and physical proximity of integrin αvβ3 and VEGFR-2 in the same melanosomes and endothelial cells. Physical proximity between proteins is critical for cell signaling and other responses (Giancotti and Ruoslahti, 1999; Giancotti, 2000). For example, physical interactions between adapter proteins, clathrin and membrane receptors are prerequisite for receptor-mediated endocytosis (Anderson, 1998; Pearse et al., 2000). The integrin αvß3 and VEGFR-2 individually and together promote tumor growth and angiogenesis (Eliceiri and Cheresh, 1999; Kasahara et al., 2000). Tyrosine phosphorylation following ligation of VEGFR-2 by VEGF is more robust and results in more pronounced angiogenic and mitogenic responses in cells that are adherent to vitronectin via integrin αvβ3 (Soldi et al., 1999; Byzova et al., 2000). Such VEGFR-2-mediated effects are blocked by an anti-ß3 integrin antibody to confirm critical implications of the integrin. Integrin αvβ3 acts down-stream of VEGFR-2 and, therefore, is a more crucial component of this cascade. These observations are strengthened by our immuno-electron microscopic data that show physical relationship between these two proteins. The expression of both the proteins at the same cellular sites in canine melanomas suggests putative molecular collaboration between them to increase survival and growth of melanocytes and endothelial cells in these tumors.

The observations do not support the hypothesis that there are differences in the expression of integrin αvβ3 and VEGFR-2 between cutaneous and oral melanomas. Accordingly, integrin αvβ3 and VEGFR-2 appear not to have direct association with diversity in biological behavior of cutaneous and oral melanomas of dogs. It is possible that angiogenesis in melanomas is regulated by some other mechanisms since a recent paper showed increased VEGF-mediated angiogenesis in ß3-knockout mice (Reynolds et al., 2002). Moreover, the individual interactions of the integrin and VEGFR-2 with other angiogenic molecules are still not fully clarified, and these interactions may potentially influence the behavior of canine melanomas.

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