Histol Histopathol (2002) 17: 347-352

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Immunohistochemical analysis of ß3 integrin (CD61): expression in pig tissues and human tumors

A. Moreno[1], C. Lucena[2], A. López[3], J.J. Garrido[1], J.M. Pérez de la Lastra[1] and D. Llanes[1]

[1]Department of Genetic, Spanish Research Council-University of Córdoba, [2]Department of Cell Biology, Faculty of Medicine, University of Córdoba and [3]Department of Medical Surgery, Faculty of Medicine, University of Córdoba, Córdoba, Spain

Summary. CD61 is a membrane glycoprotein that associates with CD41 (αIIb) to form the heterodimeric complex gpIIb/IIIa (CD41/CD61), predominantly expressed in platelets and megakariocytes. CD61 or $ß_3$ integrin also associates with αv (CD51) to form the vitronectin receptor, which is expressed in many tissues. We have used a monoclonal antibody against the porcine gpIIIa or CD61 (JM2E5) to study the distribution of this molecule in different normal pig tissues. As in humans, CD61 was broadly expressed in all tissues examined. In the kidney, strong expression of CD61 was observed in epithelial cells from renal tubules. In the testis, CD61 expression was detected in the Leydig cells. However, in liver, CD61 was weak or not detected.

Many integrins are particularly involved in tumogenicity and in tumor progression mediating cell-cell interaction. Immunofluorescence experiments using cultured human tumor HeLa cells showed nuclear and cytoplasmic staining of mAb JM2E5. Immunohistochemical analysis of human tumor sections from several organs showed a heterogeneus distribution in metastatic cases from colon and breast carcinoma. However, no staining was found in metastasis from melanoma.

Key words: $ß_3$ integrin, Immunohistochemistry, Pig tissues, Human tumors, Monoclonal antibody

Introduction

Platelet glycoproteins αIIb and $ß_3$ (CD41/CD61) are membrane proteins that associate to form a Ca^{2+}-dependent heterodimer which constitutes a member of the integrin family at the surface of the cell. In resting platelets it is constitutively expressed in an inactive state and it does not recognize soluble proteins. Platelet activation results in a conformational change from a low-affinity CD41/61 receptor to a high-affinity state, which then recognizes the plasma fibrinogen, fibronectin, von Willebrand factor, vitronectin and thrombospondin (Kieffer and Phillips, 1990).

Monoclonal antibodies (mAbs) to gpIIb/IIIa complex have proven to be a useful tool for functional and structural studies of this molecule. Animal homologues of human gpIIb/IIIa have been detected in different species. We have reported previously a murine mAb (JM2E5) to porcine CD41/CD61 (Pérez de la Lastra et al., 1997). This antibody recognizes the $ß_3$ subunit of the complex and is cross-reactive with human gpIIb/IIIa.

In humans, CD61 or gpIIIa integrin is expressed in cells from the megakaryocytic lineage and has been described in human monocytes and macrophages (Prieto et al., 1994). However, the tissue distribution of this protein in normal human tissues is not well known. In the III International Veterinary Immunology Workshop and Conference on Human Leukocyte Differentiation Antigens (Pilkington et al., 1987), two anti-gpIIIa mAbs were reactive with endothelial cells, renal glomerular and tubular cells and smooth muscle cells. Most of the mAbs anti gpIIb/IIIa from this workshop, showed reactivity with bone marrow, fetal liver, lung megakaryocytes and with a small percentage of bone marrow blasts.

The complex CD41/CD61 is not only present in normal tissue. It has also been found in 17 tumor cells lines (Chen et al., 1997) derived from different species (human, rat and mouse) and of different histological origins: skin, blood, lung, liver, kidney, cervix, colon, bladder, breast and prostate. In these tumor cells, the glycoprotein could be involved in the invasion of human melanoma cells (Trikha et al., 1997) and in the metastatic progression of prostatic adenocarcinoma (Trikha et al., 1998). Moreover, a number of studies have suggested that interactions of integrins with their ligands are associated with tumorigenicity and tumor progression.

In this study, we tested the immunochemical reactivity of a mAb (JM2E5) anti-pig CD61 to determine the distribution of this glycoprotein in different normal porcine tissues. Because integrins are involved in the growth, invasion and metastatic properties of many types

Offprint requests to: Angela Moreno López, Departamento de Genética, Campus Rabanales, Edificio C-5, 1ª planta, 14071 Córdoba, Spain. Fax: 34 957 218730. e-mail: ge1moloa@uco.es

of tumors, the expression of CD61 integrin in tumors was also investigated. Given that JM2E5 is cross-reacting with human, we studied the expression of CD61 in cortexes of primary human tumors and their metastasis.

Materials and methods

Cells and antibodies

HeLa cells were purchased from the ATTCC. JM2E5 antibody was generated in house as previously described (Perez de la Lastra et al., 1997). Rabbit anti-mouse IgG FITC-labeled antibody was purchased from Sigma Co. St. Louis, USA. Rabbit anti-mouse Ig-biotinilated antibody was purchased from DAKO Corp. Carpinteria, USA.

Cell cultures

HeLa cells were grown in DMEM (Gibco Co USA) supplemented with 10% FCS and 1% (v/v) penicillin/streptomycin (Sigma) and plated in 6-well culture plates (Corning Incorporated, Corning, New York, USA), containing sterilized coverslips and left to 60% confluence. The cultures were serum deprived in DMEM with BSA (1mg/ml). Cells were then washed twice with PBS 0.1M, pH 7.4, and fixed in paraformaldehyde (4% in PBS) for 15 min at room temperature. After washing three times with PBS, cells were permeabilized with 0.5% Triton X-100 for 30 min. After washing in PBS, coverslips were incubated with JM2E5 antibody diluted 1/20 in PBS, or with PBS as negative control, for 1 hour at 37 °C. Cells were washed again in PBS and stained with rabbit anti-mouse IgG FITC-labeled (Sigma) diluted 1/25 for 1 hour at 37 °C. Finally, coverslips were washed and mounted in PBS/glycerol (1:1).

Histological samples

Samples from porcine tissues (collected from five different pigs) and tumor specimens were placed overnight into 10% buffered formalin. Five µm paraffin-embedded sections, mounted on poly-L-lysine-coated slides, were dewaxed in xylene and hydrated by passage through graded alcohols. Endogenous peroxidase activity was inhibited by treatment with 3% hydrogen peroxide in distilled water for 30 min at room temperature. After washing with PBS, the slides were microwaved as previously described (Sierralta and Thole, 1996). Briefly, slides were immersed in 10 mM citrate buffer pH 6.0 and microwaved in an R-210a Sharp microwave oven run 5 min at medium and twice (3 min each one) at maximal power (800 w). After cooling, the sections were covered with distilled water for 20 min, washed in PBS and incubated with normal goat serum (1:20 dilution in 1% albumin-PBS) for 30 min at room temperature. After removing the serum, JM2E5 antibody or PBS-BSA (as negative control) was added for 18 hours at 4 °C in a wet chamber. The sections were washed in PBS and incubated with anti-mouse Ig-biotinilated (DAKO), diluted 1/50 in PBS-BSA, for 30 min at room temperature. After washing again in PBS, tissue sections were covered with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, USA) for 1 hour in a wet chamber, washed and then developed with 3,3'-diaminobenzidine (Sigma) (DAB: 5 mg in 10 ml PBS). Sections were counter-stained with Carazzi hematoxylin and mounted with Immu-mount (Shandom Inc. Pittsburgh, USA).

The percentage of stained cells (staining score) was evaluated as 0 (no staining); 1 (0-5 %); 2 (5-50 %) and 3 (more than 50%). Staining intensity was considered as 1 (weak-moderate) and 2 (strong).

Results

Immunohistochemical reactivity of the antibody JM2E5 specific for CD41/CD61 was tested on paraffin-embedded sections of the following normal porcine tissues: skin, intestine, testis, liver, spleen and kidney. Paraffin-embedded sections were preferred to frozen tissue sections due to their widespread use in diagnostic immunohistochemistry and for a better comparison with the distribution of CD61 in human.

Immunohistology using the anti-gpIIIa mAb JM2E5 confirmed that CD61 was abundantly distributed in pig tissues, particularly on cells of epithelial origin. Negative controls using an isotype-matched mAb were performed for each tissue. In controls, only occasional dark brown-stained cells were observed, which were eosinophils in which endogenous peroxidase activity was not fully blocked. The vascular endothelium in some organs examined was positive for pig CD61, although the constitutive expression of this integrin in endothelial cells has been controversial (Damjanovich et al., 1992; Singh et al., 2000). In skin, immunoreactivity was evident at the granular and prickle cell layers as well as in the blood vessels and glandular cells near the hipodermis (Fig. 1a,b). In contrast, pig CD61 was absent from smooth muscle cells in the dermis (not shown). Intestinal mucosa showed staining of JM2E5 in epithelial cells of the glands, leukocytes and plasma cells at the lamina propria (Fig. 1c). The testis was strongly stained for CD61, particularly the Leydig cells and surrounding connective tissue; the germinal epithelium in the seminiferous tubules showed a slight positivity (Fig. 1d) and in spleen, the staining for CD61 was located in lymphoid aggregates of the white pulp nodules (Fig. 1e). However, CD61 was absent from the abundant splenic supporting tissue (not shown). In the kidney, the staining for CD61 was strong in all epithelial cells of renal tubules (Fig. 1f), but staining of glomerular epithelium was weak (not shown)

The expression of integrins gpIIb/IIIa in cultured tumor cell lines is well documented. Therefore, we studied the expression of CD61 integrin in HeLa cells,

using the mAb JM2E5. Immunofluorescence experiments showed that HeLa cells were stained by JM2E5 antibody. A staining pattern with nuclear and cytoplasmic paranuclear expression was observed (Fig. 2).

On this basis, we questioned whether the expression of CD61 integrin observed in cultured tumor cells was also found in vivo. In this sense, immunohistochemical expression using mAb JM2E5 was investigated in formalin-fixed, paraffin-embedded surgical samples of human carcinomas of colon, breast and melanoma, as well as in their metastasis.

As shown in Table 1, normal breast tissue was not stained using mAb JM2E5 and a moderate staining for CD61 was observed in normal skin and colon mucosa (Fig. 3). Histological distribution of JM2E5 was

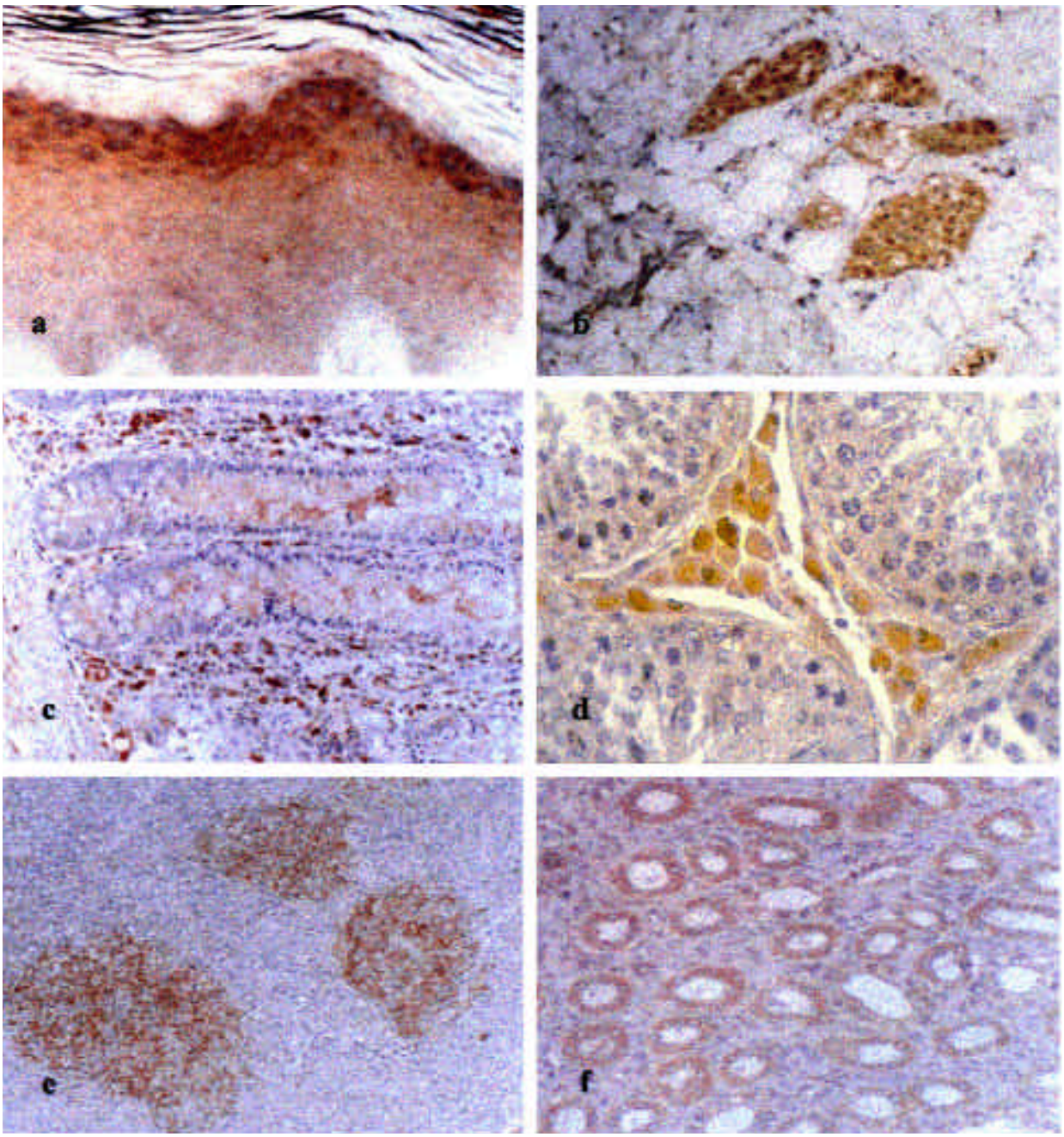

Fig. 1. Immunoreactivity of JM2E5 in porcine tissues. Staining is mainly localized at the epidermis and glandular cells in skin **(a, b)**; cytoplasmic pattern shows leukocytes, plasma cells and epithelial cells of intestinal mucosa **(c)** as well as the Leydig cells in testis **(d)**. Reactivity with lymphoid aggregates of the white pulp in spleen **(e)** and epithelial cells of renal tubules **(f)** is also seen. x 100

heterogeneous in all the different cancer cases (Table 1). Colon and breast carcinoma showed atypical glandular structures, infiltrating normal tissues and JM2E5 stained in an intracytoplasmic fashion, with a membranous pattern (Fig. 4a,c). This heterogeneous distribution of the CD61 integrin was similar in all metastatic cases (Fig. 4b,d,f). In melanoma, JM2E5 staining also had a diffuse intracytoplasmic location with a membranous pattern (Fig. 4e). However, no staining was found in the metastasis of melanoma (not shown).

Discussion

The complex of integrins CD41/CD61 is the most thoroughly studied protein on platelets, but there are few references about the distribution in tissues by immunohistochemistry. The distribution of CD61 in species other than man has not been thoroughly examined. Here we provide an analysis of the distribution of CD61 in the pig. Since platelets almost invariably adhere to all leucocytes, only immunohistochemical techniques make it possible to clearly distinguish between cells expressing the antigen and nonspecifically-stained cells. In the present study, we have used immunohistochemical techniques in order to clarify the distribution of this glycoprotein in various normal porcine tissues

Our data show a widespread expression of the CD61 antigen in different organs of swine corresponding to the pattern of tissue expression for beta 3 integrin, which is different from the pattern found when mAbs specific to CD41, the alpha IIb integrin are used. Expression of the CD41 is restricted to the megakaryocyte/platelet lineage (Jim et al., 1998), whereas CD61 is also present on macrophages, monocytes and endothelial cells. CD61 forms with αv the vitronectin receptor. Recently, the expression of $\alpha v\beta_3$ molecule has been studied in pig, dog and cattle tissues (Singh et al., 2001). Similarly to our results, the $\alpha v\beta_3$ integrin is expressed on cells of epithelial origin. Our results are also consistent with those found in humans by Soligo et al. (1989). These authors also suggest the possibility of the existence of cross-reacting epitopes on gpIIb/IIIa with LFA-3 y Mac-1 molecules, which belong to the same family of adhesion molecules. However, we did not detect LFA-3 or Mac-1 molecules by immunoprecipitation studies

Table 1. Distribution of JM2E5 in human tumor samples.

SPECIMENS	No. OF CASES	STAINING SCORE				STAINING INTENSITY	
		0	1	2	3	1	2
Normal Colon mucosa	3	0	3	0	0	--	3
Normal breast tissue	3	3	0	0	0	--	--
Normal skin	1	0	1	0	0	1	--
Colon Carcinoma	5	0	0	2	3	--	5
Liver metastasis	5	0	0	4	1	1	4
Breast Carcinoma	3	0	0	2	1	--	3
Skin metastasis	2	0	1	0	1	--	2
Bone marrow	1	0	0	0	1	--	1
Melanoma	1	0	0	0	1	--	1

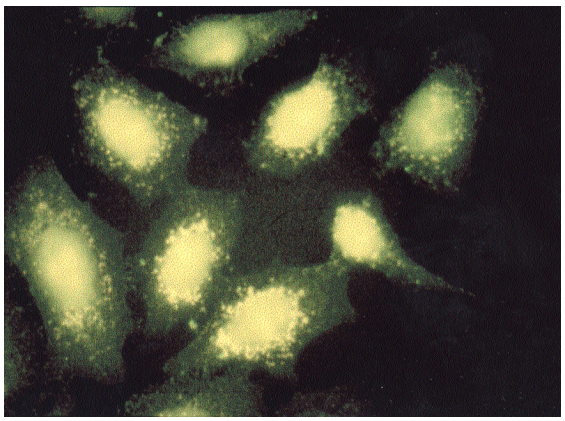

Fig. 2. Immunofluorescence in cultured HeLa cells showing the binding pattern for JM2E5. x 75

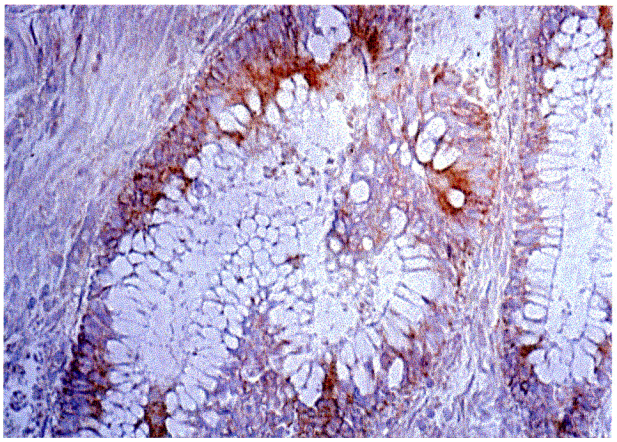

Fig. 3. Human colon mucosa. Immunoreactivity in epithelial cells is observed. x150

using the mAb JM2E5 (not shown).

In addition to the normal distribution of CD61 in the pig, we have undertaken the expression of this integrin in the cortex of human tumors using a mAb against porcine gpIIIa. Concerning the expression of gpIIb/IIIa in human tumor, several reports have been published which demonstrate the presence of adhesion molecules on tumor cell membrane. Adhesion molecules are detectable with antibodies against platelet glycoproteins and they are involved in the growth, invasion and metastatic properties of many types of tumors (Varner and Cheresh, 1996). Particularly, the integrins are involved in the process of tumor progression by mediating the adhesion of tumor cells to each other and active platelets to form tumor-platelets aggregates. The $\alpha IIb\text{-}ß_3$ receptor and P-selectin on platelets are also

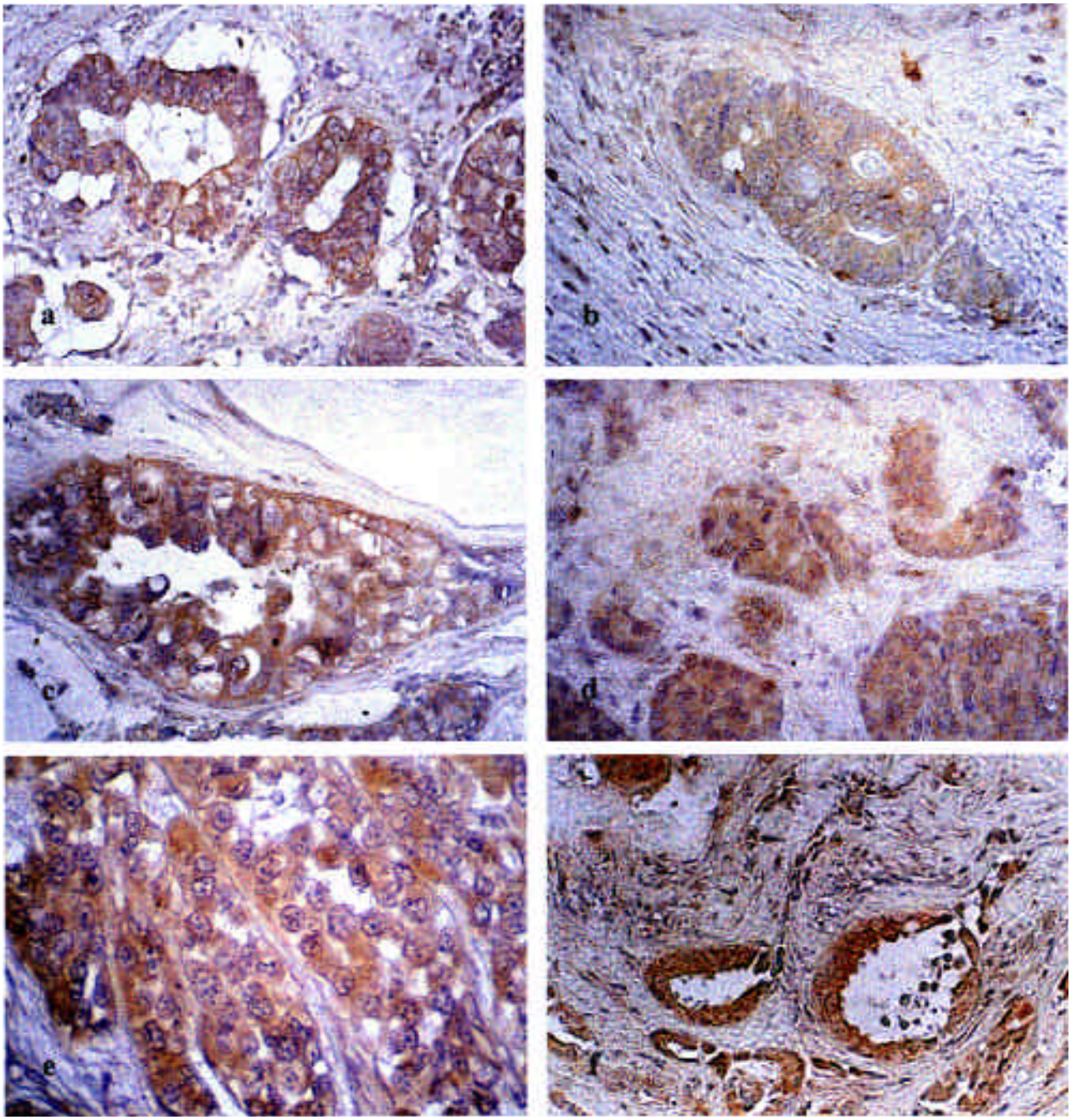

Fig. 4. Expression of JM2E5 in colon carcinoma **(a)** and liver metastasis (**b)**. Breast carcinoma **(c)** and skin **(d)** and bone marrow **(f)** metastatic cases. Melanoma **(e)**. Staining shows an intracytoplasmic location with a membranous pattern. x 150

important when the tumor cells are protected against circulating immune cells by binding to platelets (Huang et al., 1997). Several approaches aimed at inhibiting the activities of these molecules have been studied in vivo and in experimental animals for developing new therapies and, due to their specificity and unlimited availability, the most common approach has been to use mAbs, such as JM2E5, which recognize adhesion molecules (Jim et al., 1998).

The purpose of this study was to determine by immunohistochemistry whether different human tumors expressed the gpIIb/IIIa because little is known about the expression of this protein in vivo, as the majority of investigations have been carried out in cultured human tumor cell lines. To address this question, we screened breast, colon and melanome tumor tissue and the analysis indicates positive staining with JM2E5 in all these specimens, confirming the presence of alpha IIb-beta3 in vivo. So, these results suggest that this integrin is expressed in nonmegakaryocytic lineage solid tumor cells. Trikha et al. (1997) found that the high affinity gpIIb/IIIa integrin is capable of directly supporting melanoma cell adhesion and that this complex may have important consequences on the development of the metastatic phenotype in prostate cancer (Trikha et al., 1998). The latter results could be in accordance with the presence of αIIb-ß$_3$ found by us in metastasis of skin and liver. More experiments are necessary in order to confirm these results and the mAb JM2E5 can be very useful in this investigation and in cancer therapy also, because different studies (Edward, 1995; Newton et al., 1995)) have shown that neutralizing mAbs which target adhesion molecules is effective in inhibiting invasion, dissemination and/or proliferation of tumor cells in animal systems.

Acknowledgements. JMPL is a recipient of a "Marie Curie"-TMR fellowship, contract QLK3-CT-1999-51523. This work was supported by the Spanish CICYT, project BIO2000-1636-CO2-02. We are grateful to Ana Martin White for the assistance with the manuscript.

References

Chen Y., Trikha M., Gao X., Bazar R. and Portes A. (1997). Ectopic expression of platelets integrin alphaIIb-beta3 in tumor cells from various species and histological origin. Int. J. Cancer 72, 642-648.

Damjanovich L., Albelda S., Mette S. and Buck C. (1992). Distribution of integrin cell adhesion receptors in normal and malignant lung tissue. Am. J. Respir. Cell. Mol. Biol. 6, 197-206.

Edward M. (1995). Integrins and other adhesion molecules involved in melanocutic tumor progression. Curr. Op. Oncol. 7, 185-191

Huang Y., Baluma R. and Vitetta E. (1997). Adhesion molecules as targets for cancer therapy. Histol. Histopathol. 12, 467-477.

Jim Y., Wilhide C., Dang C., Li L., Li S., Villa-Garcia M. and Bray P. (1998). Human integrin beta 3 gene expression: Evidence for a megakaryocytic cell-specific cis-acting element. Blood 92, 2777-2790.

Kieffer N. and Phillips D.R. (1990). Platelets membrane glycoproteins: function in celular intereactions. Annu. Rev. Cell Biol. 6, 329-357.

Newton S., Reves E., Gralanick H., Mohla S., Yamada K., Olden K. and Akiyama S. (1995). Inhibition of experimental metastasis of human breast carcinoma cells in athymic nude mice by anti alpha5-beta1 fibronectin receptor integrin antibodies. Int. J. Oncol. 6, 1063-1070.

Pérez de la Lastra J.M., Moreno A., Pérez J. and Llanes D. (1997). Characterization of the porcine homologue to human platelet glycoprotein IIb-IIIa (CD41/CD61) by a monoclonal antibody. Tissue Antigens 49, 588-594.

Pilkington G., Hogg N., Thorsen L. and Horton M. (1987). Platelet antigens. In: Leukocyte typing III. McMichael A. (ed). University Press. Oxford. pp 733-746.

Prieto J., Eklund A. and Patarroyo M. (1994). Regulated expression of integrins and other adhesion molecules during differentiation of monocytes into macrophages. Cell Immunol. 156, 191-211.

Sierralta W. and Thole H. (1996). Retrieval of estradiol receptor in paraffin sections of resting porcine uteri by microwave treatment. Immunostaining patterns obtained with different primary antibodies. Histochem. Cell Biol. 105, 357-363.

Singh B., Fu C. and Bhattacharya J. (2000). Vascular expression of the αvß$_3$ integrin in lung and other organs. Am. J. Physiol. 278, L227-L226.

Singh B., Rawlings N. and Kaur A. (2001). Expression of integrin αvß$_3$ in pig, dog and cattle. Histol. Histopathol. 16, 1037-1046.

Soligo D., Cattoretti G., Colombi M., Polli N., Capsoni F., Rilke F. and Lambertenghi G. (1989). Bone marrow and tissue expression of gpIIb/IIIa, LFA-1, Mac-1 and gp150, 95 glycoproteins. Eur. J. Haematol. 42, 173-181.

Trikha M., Timar J., Lundy S., Szekeres K., Cai Y., Porter A. and Honn K. (1997). The high affinity alpha IIb-beta 3 integrin is involved in invasion of human melanoma cells. Cancer Res. 57, 2522-2528.

Trikha M., Raso E., Cai Y., Fazakas Z., Paku S., Porter A., Timar J. and Honn K. (1998). Role of alpha IIb-beta 3 integrin in prostate cancer metastasis. Prostate 35, 185-192.

Varner J. and Cheresh D. (1996). Integrins and cancer. Curr. Op. Cell Biol. 8, 724-730.

Accepted November 8, 2001