Role of the extracellular matrix proteins in the resistance of SP6.5 uveal melanoma cells toward cisplatin

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Abstract. Uveal melanoma is the most frequent primary intraocular tumor in the adult population. This malignancy has a high mortality rate and responds poorly to existing chemotherapy. Recently, the tumor environment has been found to exert a profound influence on drug response through cell interaction with components from the extracellular matrix (ECM). In the present study, we investigated whether individual components from the ECM may affect cell survival and/or cell death induced by the cytotoxic agent cisplatin on the SP6.5 uveal melanoma cell line. Tumor cells were shown by immunofluorescence analyses to be surrounded by the ECM proteins fibronectin (FN), type IV collagen (CIV) and laminin (LM), both at the primary and metastatic sites. Binding of SP6.5 cells to FN, LM and CIV is primarily dictated by the expression of membrane bound integrins from the ß1 family as revealed by cell adhesion assays conducted on ECM-coated culture plates. Analysis of cell death by flow cytometry demonstrated that culturing SP6.5 cells in the presence of FN, CIV and LM, substantially reduced the percentage of cells undergoing apoptosis after cisplatin treatment when compared with those seeded on a non-permissive matrix. These results suggest that adhesion of the SP6.5 uveal melanoma cells to the ECM proteins FN, CIV and LM might therefore confer resistance to the chemotherapeutic agent cisplatin. The cellular resistance induced by the ECM proteins toward cisplatin could explain in part the local recurrence of metastasis derived from uveal melanoma often observed clinically after chemotherapy.

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Introduction

Uveal melanoma is the most common type of primary intraocular tumor in the adult population (1). This malignancy is remarkable for its haematogenous dissemination, its unpredictable clinical course and its high tendency to metastasize primarily to the liver (2,3), often after a long disease-free interval (4). Chemotherapy remains the mainstay for the treatment of disseminated cancers including haematologic malignancies and metastatic solid tumors (5). Despite new treatments for primary uveal melanoma that have emerged over the past few years, the survival rate has remained unchanged (3). Metastatic uveal melanoma lesions appear to be highly resistant to most available chemotherapeutic agents presently available for the treatment of various malignancies as well as those established for the therapy of cutaneous melanoma (4,6). The use of the BOLD chemotherapy regimen (dacarbazine, lomustine, vincristine and bleomycin) combined with interferon-α was shown in the European Organization for Research and Treatment of Cancer (EORTC) multicentre study to be ineffective, despite encouraging data from the initial series (4,7). For metastatic disease localized to the liver, intra-arterial injections of fotemustine or carboplatin, or chemoembolization with cisplatin (cis-dichlorodiammine platinum; cDDP) report a response rate of 29-40% and a median overall survival of 6-18 months (4,6-8). Even if impressive initial responses to chemotherapy are seen in a significant number of patients, residual and recurrent disease refractory to further treatment could results in tumor relapse (9). Even today, the problem of multidrug resistance (MDR), either by intrinsic or acquired mechanisms, still remains a major obstacle preventing cures of many forms of cancers (5). Expression of the MDR1 gene, encoding for the efflux pump P-glycoprotein, appears to be involved in the chemoresistance of primary uveal melanoma (10). Other classical mechanisms of MDR, such as glutathione-S-transferase upregulation and topoisomerase II mutation or downregulation (8) have not been described in uveal melanoma.

Recently, evidence has accumulated suggesting that the direct interaction of cancer cells with components from their microenvironment may influence cancer cells response to chemotherapy (5). Indeed, interactions between cell surface

integrins and ECM components have been shown to account for this phenomenon of innate drug resistance termed cell adhesion mediated-drug resistance, or CAM-DR (11).

It is well recognized that loss of cell-cell or cell-ECM adhesion that lead to cell death, which is identified as anoikis, regulates apoptosis and cell survival in a wide variety of primary and transformed cells (12,13). Furthermore, many forms of cancer chemotherapy primarily exert their cytotoxic effects by inducing apoptosis (9,11,14). For example, adhesion of cells to the ECM component LM resulted in an increased resistance to chemotherapy-induced apoptosis in small-cell lung cancer (15). It has been shown that vitronectin, but not FN, two other well-known components from the ECM, protects human glioma cell lines against etoposide-induced death (16).

Very little is known about the function of MDR in the chemoresistance and tumor propagation of uveal melanoma. Besides, nothing has ever been reported about the possible contribution of CAM-DR in metastatic uveal melanoma. Here we took advantage of the uveal melanoma cell-line SP6.5, a spindle-epithelioid mixed type culture obtained from a primary tumor (17,18), to gain information on the chemoresistance conferred by the ECM. This cell line proved to be a useful model as it possesses the ability to produce tumor and liver metastasis in nude mice (19). Due to the *in vivo* infiltrative growth of uveal melanoma, these cancer cells inevitably interact with basement membrane proteins (such as laminin and type IV collagen) at the site of intra- and extravasation. Besides, SP6.5 cells were shown to secrete FN *in vitro* and *in vivo* and to organize it in complex networks (17,18). In the present study, we investigated whether individual components from the ECM may affect cell survival and/or cell death induced by cDDP, a cytotoxic agent extensively used in clinic for the treatment of several forms of cancers, including uveal melanoma, on the SP6.5 uveal melanoma cell-line. We found that several ECM proteins modulate the cDDP-induced cytotoxicity toward SP6.5 cells and that these proteins are present in the tumor environment of uveal melanoma at primary and metastatic sites.

Materials and methods

This study was conducted in accordance with our institutional guidelines and the Declaration of Helsinki, and the protocols approved by the institution's Committee for the Protection of Human Subjects.

Cell culture and reagents. All reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada), unless otherwise specified. The uveal melanoma cell line SP6.5 was maintained in Dulbecco's modified Eagle's medium (DMEM) with penicillin-streptomycin-glutamin (additives; Invitrogen, Burlington, ON, Canada), supplemented with 5% fetal bovine serum (FBS; Invitrogen) and kept under 5% CO_2 at 37˚C. The ECM component fibronectin (FN) was prepared from human plasma as previously described (20) and diluted in a solution of 33% glycerol in phosphate-buffered saline (PBS) and stored at -80˚C prior coating of the culture plates. Gel analysis indicated that the purified FN was of the appropriate molecular mass and showed no proteolytic degradation. Human placental collagen type IV and mouse laminin I were purchased from Trevigen (Gaithersburg, MD, USA) and diluted in PBS (pH 7.4) prior to their storage at -20˚C. cDDP was diluted in PBS (stock solution 2 mM) and kept frozen at -70˚C. The day of the experiment, an aliquot was thawed and appropriately diluted in PBS. The remaining stock solution was discarded.

Indirect immunofluorescence assays and nuclear DAPI staining. Fresh samples of tumor tissues obtained from 3 posterior uveal melanomas at enucleation (T88, T89 and T95) and from metastatic post-mortem liver sections (MH80) were embedded in OCT (Optimal Cutting Temperature) compound (Tissue-Tek, Bayers Canada, Etobicoke, ON, Canada), frozen in liquid nitrogen and then stored at -70˚C until used. An indirect immunofluorescence assay was performed on acetone-fixed (10 min at -20˚C) cryosections (5 µm thick) of frozen tissues as previously reported (21). Sections were incubated with the primary antibody for 45 min, followed by incubation with the appropriate conjugated secondary antibody for 30 min. Primary antibodies used included a rabbit anti-human CIV antibody (Chemicon International, Temecula, CA, USA), a mouse anti-FN antibody from a mouse lymphocyte hybridoma (clone HFN7.1, ATCC, Rockville, MD, USA), a rat anti-LM antibody (Immunotech, Westbrook, ME, USA) and a mouse anti-melanoma cells antibody (clone HMB45, Cedarlane Laboratories Limited, Hornby, ON, Canada). Secondary antibodies used included a goat FITC-conjugated anti-rat antibody (Chemicon International), and Alexa 594-conjugated chicken anti-rabbit and goat anti-mouse antibodies (Molecular Probes, Eugene, OR, USA). Cell nuclei were also labeled with Hoechst reagent 33258 (Sigma) following immunofluorescence staining. Negligible background was observed for controls (primary antibodies omitted).

Nuclear DAPI staining was conducted on cultured cells as follows: cells ($5x10^4$ cells/coverslips) were cultured on glass coverslips (25x25 mm; VWR International Ltd., Montréal, QC, Canada) in complete DMEM with FBS. Approximately 24 h after seeding, cDDP was added at either 25 or 50 µM. Cells were treated for 24 h with cDDP. Cells were then washed with PBS, fixed with 1% paraformaldehyde-PBS, and blocked with 3% bovine serum albumin (BSA) for 1 h at 37˚C. Cells were covered with 100 µl of a solution containing 3% BSA in PBS with a 1:2000 dilution of DAPI for 45 min. After 3 washes and mounting, immunofluorescence was measured using a Nikon Eclipse E800/E800M microscope with a x40 objective. Image acquisition was performed with a CCD camera (Hamamatsu, ORCA-ER) connected to a PC computer using the C-Imaging software (Compix, Inc. Cranberry Township, PA, USA).

Cell adhesion assays. Forty-eight-well plates (Falcon, Becton-Dickinson Labware, Franklin Lakes, NJ, USA) were coated with either 25 µg/ml of FN, 50 µg/ml of CIV, 25 µg/ml of LM, or with BSA and allowed to settle for 60 min at 37˚C (for FN) or overnight at 4˚C (for the other ECMs). Wells were washed and incubated with 1% BSA in PBS for 1 h at 37˚C. Subconfluent SP6.5 cells were harvested with a solution of PBS containing 0.4 w/v of sodium citrate and 0.5 M

EDTA (PBS-citrate-EDTA), washed, and ressuspended in adhesion buffer (DMEM containing 1 mM $MgCl_2$, 0.2 mM $MnCl_2$, and 0.1% BSA). The function-blocking antibodies P4C10 (ß1 blocking antibody; Chemicon International) and LM609 (αvß3 blocking antibody; Chemicon International), and the isotype control antibody C-2-10 (22) raised against the C-terminal end of the DNA binding domain of bovine poly(ADP-ribose) polymerase (PARP) were added to the SP6.5 cell suspensions ($1x10^5$ cells) at a final concentration of 1, 10 and 25 µg/ml for 30 min at room temperature and agitated on a rotator. Cells were then ressuspended in 200 µl of the adhesion buffer, added to each well and allowed to attach for 30 min at 37˚C. The non-adhered cells were removed by washing whereas adherent cells were stained for 10 min with crystal violet (1 mg/ml in 20% methanol-PBS). The wells were washed three times with PBS, and cell-associated crystal violet was eluted by addition of 100 µl of 10% acetic acid. Cell adhesion was quantified by measuring the optical density of eluted crystal violet at a wavelength of 600 nm with a microtiter plate reader.

Flow cytometric analysis. SP6.5 cells were removed from the culture dishes (Sarstedt, Montréal, QC, Canada) with PBS-citrate-EDTA solution and washed two times with serum-free DMEM containing 1% BSA and fixed with 1% paraformaldehyde-PBS for 20 min. Aliquots of fixed cells ($1x10^6$ cells) were washed twice and ressuspended in PBS (pH 7.2) containing 1% BSA. Primary antibody (1 µg) (either the P4C10 ß1 or the LM609 αvß3 blocking antibodies) was added to cell suspensions for 60 min at room temperature and agitated on a rotator. After 1 h, cells were washed twice and a dilution of 1:100 of fluorescein isothiocyanate-conjugated (FITC) human anti-mouse IgG suspended in PBS-1% BSA was added to cells and incubated for 30 min on a rotator at room temperature. Finally, cells were ressuspended in PBS and analyzed with an Epics XL flow cytometer (Epics XL; Beckman Coulter, Miami, FL).

Cytotoxicity assay. SP6.5 cells ($5x10^5$ cells/dish) were plated in 100 mm culture treated dishes (Sarstedt) and incubated in complete DMEM containing 5% FBS. Approximately 24 h after seeding, cDDP was added at a final concentration of 1, 10, 25, and 100 µM for 24 h. In order to discriminate between viable, necrotic, and apoptotic cells, a double staining with propidium iodide and Hoechst was used (23). Briefly, cells were washed with PBS and 50 µl of a 20 µg/ml solution of propidium iodide (PI) was added to the ice-cold pellets and incubation proceeded further on ice for 30 min. Approximately 25 µl of a 112 µg/ml solution of bisbenzimide Hoechst 33342 (H342) and 950 µl of a solution of 25% ethanol/PBS were added to the mixture, respectively. To estimate the cytotoxicity index in SP6.5 cells, floating cells were added to the trypsinized adherent cells and were treated together. Cells were analyzed and acquisition of data for 10^5 events was performed using an Epics XL flow cytometer. The distribution and differentiation of vital, apoptotic, and necrotic cells were analyzed from dual parameters histograms using an Epics® Elite flow cytometry workstation version 4.5 software. In all assays, a negligible amount of necrotic cells was reported.

Cell cycle analysis. SP6.5 cells ($5x10^5$ cells/dish) were plated in 100 mm culture treated dishes (Sarstedt) in the presence of 25 µg/ml of FN, 50 µg/ml of CIV, 25 µg/ml of LM or 1% BSA, which was used as a negative control, in DMEM containing no FBS. Approximately 48 h after seeding, both attached and detached cells were collected, fixed with 70% cold ethanol, and stored at -20˚C overnight. Fixed cells were washed with PBS and incubated with PBS containing RNase (100 µg/ml) and PI (10 µg/ml) at room temperature in the dark for 30 min. The DNA content of the cells was analyzed using flow cytometry and acquisition of data for 10^5 events was performed using an Epics XL flow cytometer. The distribution of SP6.5 cells within each phase of the cell cycle was analyzed from dual parameters histograms using an Epics Elite flow cytometry workstation version 4.5 software.

Apoptosis assay. SP6.5 cells ($5x10^5$ cells/dish) were plated in 100 mm culture treated dishes (Sarstedt) in the presence of either 25 µg/ml of FN, 50 µg/ml of CIV, 25 µg/ml of LM or 1% BSA, which has been used as a negative control in these experiments. Cells were incubated in complete DMEM, without FBS. Approximately 24 h after seeding, cDDP was added at either 10 or 25 µM and incubated for an additional 24 h. Discrimination between viable, necrotic, and apoptotic cells was performed as detailed above. In all assays, negligible amount of necrotic cells were reported.

Statistical analyses. Statistical comparisons of the influence of ECM components (FN, CIV and LM) versus BSA on the cDDP-treated SP6.5 cells were performed using a one-way analysis of variance followed by *post hoc* analysis with the Fisher probability of least significant difference test.

Results

Fibronectin, type IV collagen and laminin are present in the ECM-rich environment of the uveal melanoma cells. To determine the composition of the ECM that forms the dense uveal melanoma stroma, either directly produced within the tumor or deposited from the plasma, immunofluorescence analyses were conducted on human primary ocular melanomas isolated from three different patients (T88, T89, and T95) or on a liver section from a patient that developed liver metastases (MH80). As expected, a strong labeling for the various ECM components that are usually found in the basement membrane (CIV, red; LM, green) were identified around tumor-associated blood vessels (Fig. 1). There are, however, some differences in the relative expression of ECM proteins between the three primary tumors and the liver metastasis. Indeed, FN, CIV, and LM staining throughout the three primary tumors (T88, T89 and T95) exhibited a high heterogeneity from diffuse, weak, to strong staining. Both T88 and T95 yielded a strong but diffuse FN staining all over the entire section (Fig. 1a and c), whereas it was localized primarily around vascular structures in the T89 tissue (Fig. 1b). All three primary tumors stained strongly for CIV all over the tissue section and especially around vascular structures (Fig. 1e-g). LM staining was strong but localized around vessels for the T88 and T89 tumor sections (Fig. 1i and j).

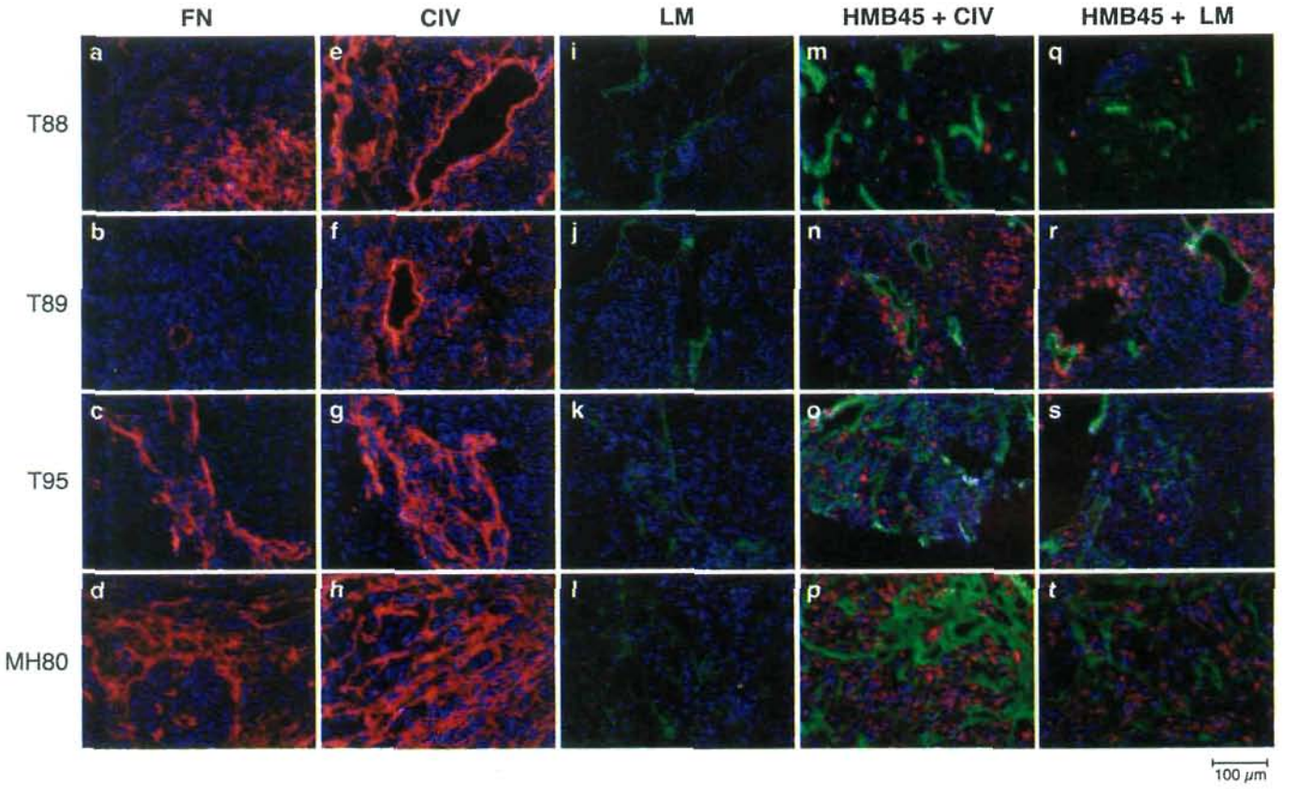

Figure 1. ECM components found in the tumor stroma of uveal melanoma. Indirect immunofluorescence staining of ECM proteins adjacent to uveal melanoma cells *in vivo*. Antibodies directed against fibronectin (FN, a-d; red fluorescence), type IV collagen (CIV, e-h; red fluorescence), and laminin (LM, i-l; green fluorescence) were used on cryosections from three primary tumors (T88, T89, and T95) and one metastatic liver section (MH80). Double-staining of uveal melanoma cells (red fluorescence) with either CIV (green fluorescence; m-p) and LM (green fluorescence; q-t) is also shown. Nuclei were counterstained with Hoechst in all pictures (blue fluorescence).

LM staining is comparable to that of FN for the T95 specimen in term of intensity and localization (Fig. 1k). On the other hand, the presence of extensive and strong staining for FN, CIV, and LM were seen throughout the metastatic sections (Fig. 1d, h and l) compared to the primary tumors specimens.

To evaluate whether the ECM proteins are closely associated with the tumor cells, tumor tissues were labeled with the HMB45 antibody, a specific marker for melanoma cells (24). Double-staining of both melanoma cells (in red in Fig. 1m-t) and CIV (in green in Fig. 1m-p) provided evidence that neither are closely associated (Fig. 1m-o), while the liver metastatic tissue shows a high degree of CIV expression in areas where HMB45-positive cells are embedded (Fig. 1p). A very similar staining pattern was also observed with LM, high level of LM expression being found around tumor cells in the metastatic foci from the liver tumor (Fig. 1t) but not from primary tumors (Fig. 1q-s). We therefore concluded that *in vivo*, FN, CIV and LM are present in the ECM-rich microenvironment that surrounds the uveal melanoma cells, with an enrichment of matrices in the immediate vicinity of the cancer cells from the metastatic liver sections.

Binding of SP6.5 cells to ECM proteins FN, CIV, and to LM is primarily mediated through ß1 integrins. It is well established that ECM elicits a wide variety of cell signaling pathways and that most of these effects are primarily mediated by membrane-bound integrins (25). Cellular binding to FN, CIV, and to LM can be mediated by several integrin receptors, most from the ß1 integrin family (26). Studies have suggested that the binding of ß1 integrins to ECM in some solid tumor types lead to an apoptosis-protecting effect (27,28). In order to determine whether ß1 integrins promote adhesion of uveal melanoma to these various ECM components, inhibition of adhesion assays were performed *in vitro*. For this purpose SP6.5 cells were preincubated with a blocking antibody (P4C10) raised against the ß1 subunit, and then plated on FN, CIV, and LM-coated culture plates. Cells unexposed to the P4C10 antibody were also used as a control. As shown in Fig. 2A, while a low concentration of P4C10 (1 μg/ml) inhibited the adhesion to both FN and LM by 50- and 70%, respectively, it virtually abolished the adhesion to CIV. The use of a higher concentration of the ß1 blocking antibody (either 10 or 25 μg) inhibited up to 75% adhesion to FN and totally abolished adhesion to both CIV and LM. Indeed, SP6.5 cells were shown to express high levels of membrane bound ß1 subunit through flow cytometry (Fig. 2B). The remaining 25% of cells attached to FN when the P4C10 antibody is used can be explained by the fact that adhesion to FN might also be mediated through non-ß1 integrins. As binding to both FN and LM can also be mediated through the αvß3 integrin, we repeated the same experiment using a blocking antibody (LM609) directed against this integrin. As

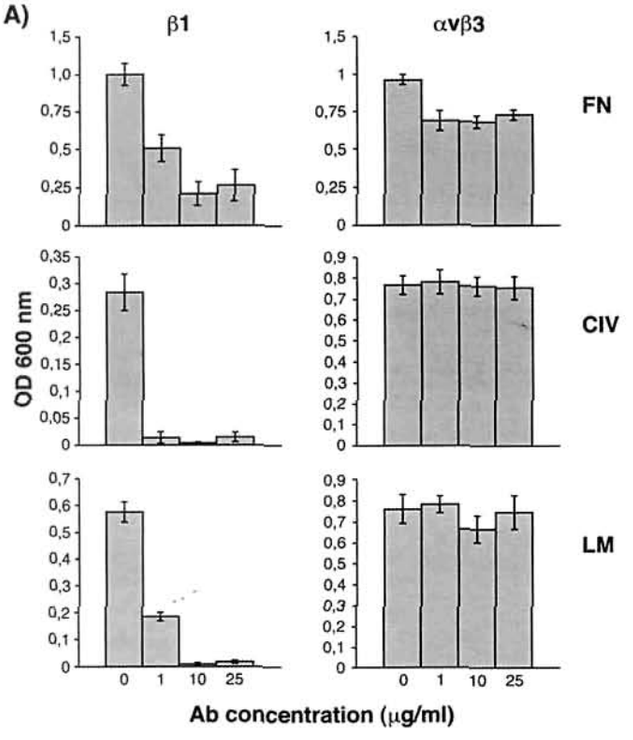

Figure 2. Integrin-mediated adhesion of SP6.5 tumor cells to FN, CIV and LM. (A), Adhesion of SP6.5 cells to FN, CIV and LM. Cells were incubated either alone (0) or with increasing concentrations (1, 10, and 25 μg) of a blocking antibody directed against either the ß1 integrin subunit (P4C10) or the αvß3 integrin (LM609; see Materials and methods), or with the isotype control antibody C-2-10 (data not presented). Cells were then seeded on either FN-, CIV- or LM-coated culture plates. Non-adherent cells were washed away and adhesion was determined by measuring the optical density of eluted crystal violet at a wavelength of 600 nm. Cells seeded on BSA coated wells were used as a negative control. Values are expressed as means ± SE of three independent experiments done in triplicate plates. Values shown on the graphs are the results of one representative out of three experiments. (B), Cell surface expression of both the ß1 integrin subunit and the αvß3 integrin was determined by incubating the SP6.5 cells with either the ß1 P4C10 or the αvß3 LM609 antibodies, respectively (solid line), followed by a further incubation with the FITC-conjugated secondary antibody. The isotype C-2-10 anti-PARP antibody was also used as a negative control (dotted line). The histogram shown gives relative fluorescence as a logarithmic scale of 4 log cycles in the X-axis and the cell number as a linear scale in the Y-axis. Data of one from three similar experiments are presented.

shown on Fig. 2A, 1 μg LM609 antibody reduced adhesion of SP6.5 cells to FN by approximately 25% whereas it had no influence on cell adhesion to both CIV and LM, even when used at 25 μg. Indeed, SP6.5 cells were found to express this receptor through flow cytometry (Fig. 2B) although to a lower level than that observed for the ß1 subunit. We therefore conclude that both the ß1 integrin subunit and the αvß3 integrin are expressed in the SP6.5 uveal melanoma cell line and that adhesion of these cells to FN, CIV, and LM is primarily dictated by ß1 integrins.

Table I. Cell cycle phase distribution 48 h after exposure to ECM proteins.

Conditions	% G_1	% S	% G_2/M
FBS	43.0±0.6	42.7±0.9	14.0±1.2
BSA	61.7±2.4[b]	22.0±2.0[b]	16.3±0.7
ECM proteins			
FN	59.7±0.7[b]	25.7±1.3[b]	14.7±1.8
CIV	57.3±2.0[a]	27.0±1.7[b,c]	15.7±1.8
LM	57.0±3.0[a]	31.0±1.5[a,d]	12.0±1.5

Flow cytometry analysis of the cell cycle with propidium iodide staining. SP6.5 cells were plated on ECM proteins, harvested and analyzed to distinguish populations of cells in different phases of the cycle. [a]P<0.0005 and [b]P<0.0001 vs. FBS; [c]P<0.05 and [d]P<0.005 vs. BSA. Values are expressed as a mean ± SE of three independent experiments.

Effect of ECM proteins on the distribution of cell cycle phases. Interactions between cells and the ECM components exert profound effects on cell survival, growth, and differentiation (29). To further understand the effect of FN, CIV and LM on the proliferative state of the SP6.5 cell line, the cell cycle distribution was analyzed by flow cytometry with PI staining. SP6.5 cells cultured for 48 h in complete DMEM containing FBS exhibited a typical distribution of proliferating cells constituted of 43.0±0.6% G_1, 42.7±0.9% S-phase and 14.0±1.2% G_2/M-phase cells (Table I). In contrast to cells cultured in the presence of FBS, SP6.5 cells plated on BSA in FBS-free medium had a significantly higher proportion of cells in G_1 phase (61.7±2.4) and a lower proportion of cells in S-phase (22.0±2.0). Cells plated on FN, CIV or LM had very much the same proportion of cells in G_1 phase (59.7±0.7, 57.3±2.0, and 57.0±3.0%, respectively) as those obtained with cells grown on BSA. However, a significant increase in the proportion of cells in S-phase was observed when SP6.5 cells were grown on both CIV and LM (27.0±1.7, and 31.0±1.5% respectively) but not on FN (25.7±1.3) compared with cells grown on BSA. For either condition, negligible amount of cell death was observed.

Sensitivity and morphological changes of SP6.5 cells toward cisplatin-induced apoptosis. To investigate the sensitivity of SP6.5 cells toward apoptosis-induced chemotherapy, cells were treated with cDDP for 24 h, and the percentage of cells undergoing apoptosis was determined by cytotoxicity assays.

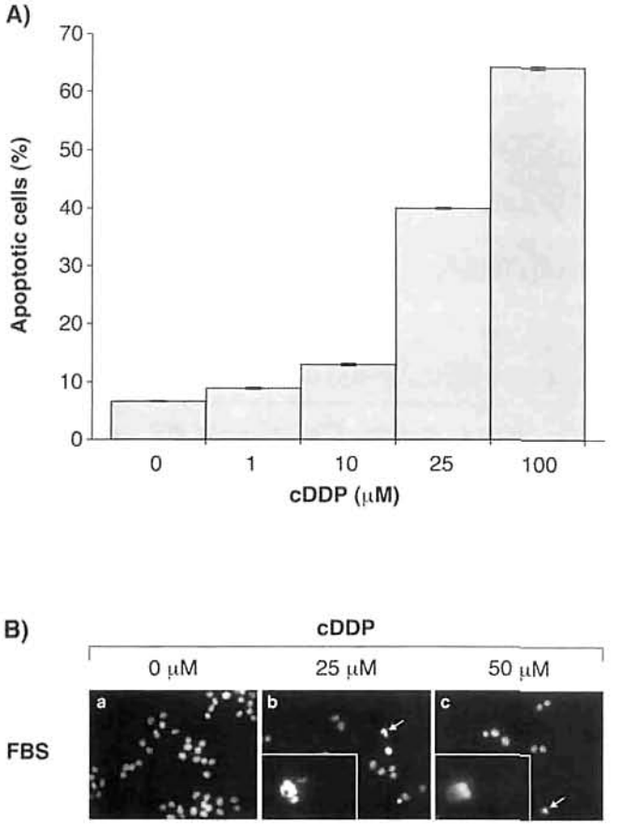

Figure 3. Effect of cisplatin-induced apoptosis on SP6.5 cells. (A), SP6.5 cells were incubated for 24 h with increasing concentrations of cDDP (0-, 1-, 10-, 25-, and 100 μM) and apoptosis estimated by flow cytometry. Hoechst fluorescence (525 nm) versus PI fluorescence (610 nm) was analyzed and the percentage of cells undergoing apoptosis was reported in a histogram. Values are expressed as means ± SE. Data of one from three similar experiments are presented. (B), Morphological assessment of cDDP-induced apoptosis of SP6.5 cells. SP6.5 cells were cultured in FBS-containing medium with either no (0 μM), or 25- and 50 μM cDDP for 24 h. Changes in nuclei morphology were then followed by DAPI staining under fluorescence microscopy. Arrows indicate small, condensed, and fragmented nucleus typical of apoptotic cells. Magnification, x400.

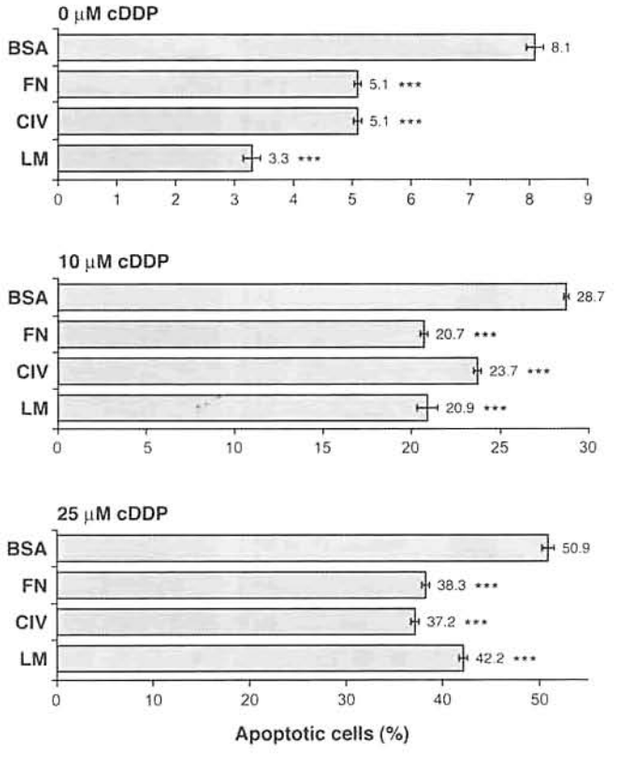

Figure 4. Apoptosis assay. Effect of FN, CIV and LM on cisplatin-induced apoptosis in SP6.5 cells. Cells were seeded on either BSA, FN-, CIV- or LM-coated culture plates and exposed to either no (0 μM), or 10- and 25 μM cDDP for 24 h. The percentage of apoptotic cells was then determined by flow cytometry. Hoechst fluorescence (525 nm) versus PI fluorescence (610 nm) was analyzed and the percentage of cells undergoing apoptosis was reported in a histogram. ***P<0.0001 vs. BSA. Values are expressed as means ± SE of triplicate plates for each of six experiments and a representative figure is shown.

As shown on Fig. 3A, cisplatin induced a dose-dependent increase of apoptosis, with an EC_{50} of approximately 25 μM. cDDP was therefore used at concentrations of 10, 25 and 50 μM for the following experiments. The apoptotic morphological changes of SP6.5 following cDDP treatment were visualized following DAPI staining to demonstrate the presence of nuclear shrinkage. As seen in Fig. 3B, exposure of SP6.5 cells to 25 and 50 μM cDDP for 24 h resulted in marked morphological nuclear changes. At a higher magnification, many cells have a small, condensed, and fragmented nucleus indicative of apoptosis.

Cell adhesion-mediated drug resistance in uveal melanoma SP6.5 cells. Recent studies have indicated that tumor cell adhesion to a specific matrix is capable of modulating resistance to drug-induced apoptosis in some type of cancer cells, such as myelomas, glioma and small cell lung cancer cells (11,16,28). We examined whether the same is true for uveal melanoma cells. Fig. 2 shows that SP6.5 cells attach to FN, CIV and to LM and as judged by cytotoxicity assays and by morphologic changes (Fig. 3), the addition of the chemotherapeutic agent cDDP induced a concentration-dependent increase in SP6.5 cell death. In order to test the hypothesis that ECM proteins might protect uveal melanoma cells from chemotherapy-induced apoptosis, we treated SP6.5 cells with cDDP in the presence or absence of various ECM proteins and performed an apoptosis assay. Cells were pre-attached to FN, CIV, LM or BSA coated dishes for 24 h and treated with 0, 10 or 25 μM cDDP for 24 h. Fig. 4 shows that in the absence of cDDP, SP6.5 cells (which corresponded to approximately 8% of the cells plated on BSA coated culture plates), were partially but significantly protected from apoptosis when cultured on FN, CIV or LM coated plates (25-, 25-, and 59% reduction in the relative number of apoptotic cells, respectively). These ECM components also conferred a significant protective effect to SP6.5 cells when further challenged with either 10- or 25 μM cDDP (28-, 17-, and 28% reduction in the relative number of apoptotic cells exposed to 10 μM cDDP on FN, CIV, and LM, respectively; 25-, 27-, and 17% reduction of apoptotic cells exposed to 25 μM cDDP on FN, CIV, and LM, respectively). As expected, cDDP forced a dose-dependent increase in apoptosis. Indeed, the proportion of apoptotic cells measured in SP6.5 cells attached on BSA, which did not protect cells from cDDP-

induced apoptosis, increased from approximately 8% in the absence of cDDP to 29% and 51% when exposed to 10- and 25 μM cDDP, respectively. These results suggest that matrix attachment provides a protection against cDDP induced-apoptosis in uveal melanoma cells and attachment-induced survival signaling may take place when uveal melanoma cells are adhered on the ECM matrix components FN, CIV and LM.

Discussion

Primary tumor growth and rate of progression toward metastasis are key determinants in the clinical outcome of patients with cancer. It is now well established that tumor progression requires a continually evolving network of interactions between neoplastic cells and the ECM, which, depending on its context, can actively regulate cellular processes such as growth, death, adhesion, migration, invasion, gene expression, and differentiation (30). It is well documented that metastatic uveal melanoma are highly resistant to existing chemotherapy. Because the interaction of tumor cells with the ECM is a prerequisite for tumor progression, we hypothesized that the presence of ECM components in the environment of uveal melanoma cells could explain in part the cell's resistance toward chemotherapeutic agents. This study showed that uveal melanoma cells *in vivo* are surrounded by a stroma of ECM at both primary and secondary metastatic sites. Furthermore, we demonstrated through apoptosis assays, that SP6.5 cells in direct contact with immobilized FN, CIV and LM are less sensitive to apoptosis induced by cisplatin treatment.

Thus far, the exact nature and function of ECM deposition in uveal melanoma has not been elucidated (31). The morphological properties and functional implications of the ECM pattern of formation in uveal melanoma have been extensively discussed in the context of the existence of blood-conducting channels lined by melanoma cells accompanied by the deposition of ECM components and their essential importance for tumor growth and metastasis. Until now, very little has been reported in the literature concerning the implication of ECM in the context of cell survival after chemotherapy in uveal melanoma. The distribution of the various ECM components in uveal melanoma as reported in the present study seems to be heterogeneous between different tumors, but also within the tumor itself. These observations correlate with those of other studies (32). However, and despite the results presented hereby, it remains unknown whether the ECM components are deposited by stromal or uveal melanoma cells, or both.

Many chemotherapeutic drugs, like cisplatin, contribute to anti-tumor activity by inducing apoptosis (33). The importance of understanding the role of ECM and its interaction with tumor cells has been further emphasized by numerous studies showing that deposition of matrix components and their interaction with nearby cells are of first importance in tumor progression (34). Most studies on multidrug resistance have primarily focused on isolated cancer cells with little consideration being given to the extracellular milieu (9). In this regard, we hypothesized that ECM proteins, such as FN, CIV and LM, which are secreted in the immediate environment of uveal melanoma cells *in vivo*, may protect these tumor cells from the apoptotic death induced by chemotherapeutic stresses. We demonstrated the presence of these components in the area surrounding the tumor cells, suggesting that they could influence the way these cells respond to chemotherapy. *in vitro*, the binding of SP6.5 uveal melanoma cells to FN, CIV and to LM reduced the cytotoxicity of the chemotherapeutic agents cisplatin. This may explain at least in part the inefficiency of cDDP in the treatment of patients with uveal melanoma that progressed to liver metastasis (35), the median survival rate being extended of only a few months at the most (4,8). Despite their anchorage-independence (17), binding of SP6.5 cells to ECM proteins FN, CIV and LM clearly conferred an increased protection toward chemotherapy-induced apoptosis.

Integrins regulate many intracellular signaling pathways including tyrosine phosphorylation and inositol lipid metabolism that are central to regulation of essential effectors of apoptosis (9). It is well known that integrin-mediated cell attachment protects normal mammary epithelial cells from apoptosis (13), and the mechanisms underlying integrin-mediated survival signaling have started to become uncovered. It is less clear, however, what role integrin signaling has in the regulation of cell survival and apoptosis in uveal melanoma cells. ß1 integrins mediate cell-ECM or cell-cell interactions and can transmit multiple regulatory signals (5,9,11,28,36). ß1 integrins are essential for normal development, and disruption of the cell-matrix interaction causes apoptosis of normal cells, identifying ß1-integrins as survival factors. Recently, the role of ß1-integrins in chemotherapeutic drug-induced apoptosis has been examined (36). Zhang *et al* (36) showed that overexpression of ß1 integrins confers resistance to apoptosis in hepatoma cells via a MAP-kinase dependent pathway and this ß1-integrin mediated signaling from the ECM to hepatoma cells may contribute to chemotherapy resistance.

The enhanced cell survival conferred by the ECM proteins FN, CIV and LM against drug-induced apoptosis in uveal melanoma cells could be mediated by the ß1 integrins, as ligand recognition by some members among this family of receptors was shown to inhibit apoptosis in numerous studies (11,27,28,37). Indeed, adhesion of small cell lung cancer cells *in vivo* to ECM that includes FN, LM and CIV was reported to confer resistance to apoptosis induced by etoposide, cDDP and doxorubicin. This resistance was shown to depend on ß1 integrins as it was completely abrogated by a function-blocking ß1 integrin antibody (28). Through the use of adhesion assays, we demonstrated that SP6.5 cells use primarily ß1 integrins to attach to FN, CIV and LM. Based on these results and on the previous finding that CAM-DR is primarily a ß1 integrin-mediated process (11,27,28,37), we speculated that SP6.5 cellular resistance toward cDDP is similarly mediated by ß1 integrins. Furthermore, SP6.5 cells use to a lesser extent the αvß3 integrin in order to bind FN (Fig. 2A). This integrin is critical for the skin melanoma aggressive behavior and also plays a role in reducing apoptosis following interaction of these cancer cells to endogenous ligands such as proteolyzed collagens (12,38-40). At this point, it is not known whether αvß3 protects cells from exogenous insults such as chemotherapeutic agents.

It has been well documented that progression through the G_1 phase of the cell cycle by mammalian cells requires growth factors- and ECM components-induced signal transduction. In eukaryotes, cell proliferation is regulated by external signals, such as the availability of growth factors and nutrients and by internal signals, such as those sensing cellular integrity (41). Culturing SP6.5 cells on FN, CIV, LM and BSA caused an accumulation of cells at the G_1 checkpoint significantly different from that obtained when SP6.5 cells are grown in the presence of mitogens (FBS condition) with a negligible amount of cell death. These results suggest that survival signaling is primarily dictated by the substrate on which SP6.5 cells are attached, a process clearly distinct from the mitogens induced cell survival that is typical of cells grown in FBS-containing culture medium. The significantly higher proportion of SP6.5 cells in S-phase on CIV- and LM-coated wells indicates that these matrices are more permissive for DNA synthesis than FN or BSA. As in the case of cellular stresses created by the lack of mitogens, damage to cellular components (such as DNA damage following cisplatin treatment) activate a checkpoint that arrests the cells in G_1; this withdrawal from the cell cycle might be temporary or permanent (41). It is likely that the combination of both stresses (the lack of mitogens and the DNA damaging agent cDDP) will alter more deeply the ability of the SP6.5 cells to progress through the cell cycle. In such instance, cell's attachment to ECM components like FN, CIV and LM may somehow contribute to weaken these stress factors as these components provide a survival advantage to the cell (as suggested by the results from Fig. 4).

The results presented in this study highlight a possible function for CAM-DR in the resistance of uveal melanoma cells against chemotherapy. The evidence for the involvement of CAM-DR, following its discovery in multiple myeloma cell lines, has been found in a large number of other human cell types (37). In recent years, our understanding of the tumor microenvironment has led to the suggestion that the targets of therapy should include, along with the tumor cell, the microenvironment that sustain the cell itself (27). The implication of the CAM-DR mechanism in resistance to drug-induced death in uveal melanoma and ultimately the identification of the molecular mechanisms of action of this phenomenon offers the potential of providing novel therapeutic targets to improve the response to chemotherapy and the impact on survival in cancer patients.

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