Abstract. Background: During the complex process of melanoma cell detachment from the primary tumor and subsequent metastasis, different cell surface proteins are involved in tumor cell interactions with extracellular matrix (ECM) components and surrounding cells. Melanoma cells are able to vary their expression patterns of adhesion proteins and proteases or peptidases in different stages of tumor development. Materials and Methods: This paper describes the detection of intercellular adhesion molecule-1 (ICAM-1) and aminopeptidase N (APN) on melanoma cells by immunoluminescence. The marker enzyme horseradish peroxidase (HRP) of the secondary antibody (Ab) was used to release luminescence. In addition, APN was detected on intact cells with an activity assay using alanine-p-nitroanilide (Ala-pNA) as substrate including inhibition experiments. Results: The cell surface expression of ICAM-1 and APN on melanoma cells was shown by indirect labelling using two different antibodies for each antigen. The activity and inhibition assays confirmed the immunoluminescent result for APN. Conclusion: Both cell surface proteins have a strong impact on tumor cell behaviour and are typical cell markers indicating a high metastatic tumor stage in melanoma. The extensive characterization of melanoma cells offers the possibility for therapeutic approaches with more than one target molecule. Moreover, increasing insights into the components involved in signal transduction could help to develop specific reagents that inhibit tumor-specific pathways.

Abbreviations: APN, aminopeptidase N; ICAM-1, intercellular adhesion molecule-1; Ala-pNA, alanine-p-nitroanilide; ECM, extracellular matrix; EDTA, ethylenediamine tetra-acetic acid Na2 salt; IgG, immunoglobulin G; FCS, fetal calf serum; mAb, monoclonal antibody; HRP, horseradish peroxidase.

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On the other hand, melanoma cells are able to shed membrane bound ICAM-1 to form sICAM-1 that might protect the tumor cell against cytotoxic T-cell attack by blocking the LFA-1 bound on the T-cell membrane (13). The problem in understanding melanoma cell behaviour, especially in the steps of intra- and extravasation, is the dynamics in the temporary up- or down-regulation of receptors and ligands (counter receptors) on both endothelial and tumor cells (14, 15).

Aminopeptidase N (CD13) is a zinc-dependent ectoenzyme normally present in intestine, kidney and other tissues. Extensive summaries of the role of APN in tissue invasion by various tumor cells were given by Nanus et al. (16) and Noren et al. (17). A recent review of the function, regulation and clinical aspects of APN was published by Lendeckel et al. (18). The authors hint at the first evidence suggesting a role of APN in signal transduction. Additionally, APN isolated from a melanoma cell line was analyzed for its enzymatic characteristics (19) and the biochemical and functional role of APN in melanoma cells was described in detail (20-22). Melanoma cell-surface APN was shown to be involved in type-IV collagen degradation and Matrigel invasion. In addition, an increased lung colonizing potential was shown in human melanoma cell transfectants that expressed very high levels of APN. APN is not expressed by normal melanocytes but it becomes increasingly prevalent with progressive tumor development (sequence: dysplastic naevi, melanoma in situ, invasive melanoma, metastatic melanoma).

Materials and Methods

Antibodies. All mAbs were of the IgG 1 type. Anti-CD54 (15.2) (Calbiochem/Merck Biosciences, Bad Soden, Germany); anti-CD54 (HA58), anti-CD13 (WM15) (Pharmingen, Hamburg, Germany); anti-CD13 (SJ1D1) (Coulter-Immunotech, Marseille, France); conjugates: anti-mouse-IgG (Fab)-HRP (Boehringer Mannheim, Germany). Normal mouse-IgG 1 (Sigma, Deisenhofen, Germany); anti-CD54 (15.2) (Pharmingen, Hamburg, Germany); H-Ala-pNA (Bachem, Heidelberg, Germany); 6-hydroxy-benzothiazole, 1,10-phenanthroline, Luminol (3-aminophthal hydrazide) (Boehringer (HA58), anti-CD13 (WM15) (Pharmingen, Hamburg, Germany); anti-CD13 (SJ1D1) (Coulter-Immunotech, Marseille, France); conjugates: anti-mouse-IgG (Fab)-HRP (Boehringer Mannheim, Germany). Normal mouse-IgG 1 (Sigma, Deisenhofen, Germany) and normal goat-IgG (Boehringer Ingelheim, Germany) were used as negative controls and for blocking, respectively.

Reagents. Luminol (3-aminophthal hydrazide) (Boehringer Mannheim, Germany); 6-hydroxy-benzothiazole, 1,10-phenanthroline (Sigma, Deisenhofen, Germany); H-Ala-pNA (Buchem, Heidelberg, Germany).

Cell line. The human melanoma cell line IGR 1 was supplied by W. Wohlrab (Halle, Germany) and originally delivered from C. Aubert (23). The cell line was cultured in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with L-glutamine, D-glucose, sodium pyruvate (Gibco, Life Technologies), 10% FCS, and contained penicillin, streptomycin and amphoterin B (Gibco). The cells were maintained at 37°C in a 10% CO2 atmosphere.

Cell labelling. Cells were grown to near confluence, harvested with EDTA/trypsin and washed with PBS (10 mmol/L phosphate, 150 mmol/L NaCl, pH 7.4). To reconstitute cell surface proteins, cells were resuspended and maintained in DMEM. For the detection with specific mouse mAbs, cells were washed thoroughly and incubated in PBS containing normal goat-IgG to block IgG and Fc receptors. After washing, the cells were incubated with the appropriate dilutions of the various Abs (60 min, 4°C, with shaking) followed by washing the cells twice. Subsequently, the cells were incubated with the anti-mouse-HRP conjugate (45 min at 4°C, with shaking) and after washing twice they were subjected to the luminescent test.

Luminescence. Aliquots of the cell suspension (100 µL, 5x10⁵ - 1x10⁶ cells) were mixed with 200 µL of the substrate solution (50 mmol/L phosphate buffer, pH 7.5; luminol/H₂O₂/6-hydroxybenzothiazole) (24) and the light emission was measured as intensity (relative light units: RLU/s) 30 s after the initiation.

APN activity. The peptidase activity assay was carried out as described by Laube et al. (5). Briefly, tests were performed with intact cells which were freshly harvested and washed thoroughly. After 45 min preincubation (2x10⁶ cells/0.5 mL PBS, pH 7.4) in the presence or absence of different effectors (inhibitors: 1,10-phenanthrolines, EDTA: 25°C; anti-CD13 Abs: 4°C) 0.5 mL substrate solution was added (final concentration of Ala-pNA: 1.5 mM in PBS). Cells were allowed to react 20-30 min at 37°C with gentle shaking. The amount of p-nitroaniline formed was measured in the supernatant by reading the absorbance at 405 nm. Each test was run in duplicate.

Instrumentation. Luminescent detection was performed with the luminometer Lumat LB 9501 (Berthold, Bad Wildbad, Germany) and the colorimetric assay using the Zeiss spectrophotometer Spekol UV-VIS 3.01 (Carl Zeiss Jena, Germany).

Cell viability was assessed by exclusion of trypan blue.

Results

The results obtained by immunolabelling are shown in Figure 1. Both antibodies to ICAM-1 gave signals with a similarly strong intensity. The anti-APN antibody WM15 was found to have an anti-catalytic effect on APN whereas the SJ1D1 antibody did not (25). The signal intensity of about 15000 RLU/s for anti-APN (WM15) is significantly above background, as compared to the controls. However, this Ab always exhibited a distinctly lower signal intensity compared to the anti-APN (SJ1D1) antibody. The same ratio of the signal intensities was found for two anti-uPA antibodies (4) detecting the region of the active site and an epitope outside the active site, respectively.

Results for the immunoluminescent controls (non-specific binding; isotype control: 1000-2000 RLU/s and conjugate control: ≤1000 RLU/s) were in the same range as previously reported (4). Figure 2 shows the results of the APN enzyme activity measurements with and without inhibitors. Detection of the APN activity with Ala-pNA as substrate is a reliable method (5, 26, 27). Chelating reagents such as EDTA and 1,10-phenanthroline inhibit APN as would be expected for most zinc metallopeptidases. Both inhibitors reduced the APN activity.
activity in a concentration-dependent manner, but despite lower concentrations used, 1,10-phenanthroline (0.5-1.5 mM) inhibited to a greater degree than EDTA (1-2 mM). Furthermore it was shown that both anti-APN antibodies caused a moderate inhibitory effect. However, while APN activity was not significantly inhibited by the SJ1D1 Ab clone, the WM15 Ab clone reduced the APN activity by up to 15%.

**Discussion**

Experiments have shown that cell surface proteins can be detected by immunoluminescence (4-8). In addition, analysis of cell-bound protease or peptidase activity was shown to be of particular importance for the functional characterization of tumor and non-tumor cells.
This investigation was performed to extend the characterization of the melanoma cell line IGR1. Previously, these melanoma cells were characterized by CD44(v5), uPA and the uPA receptor (uPAR). Numerous investigations have described the adhesion protein CD44 and its isoforms as well as the uPA/uPAR complex as multifunctional factors in tumor growth and metastasis. The additional definition of melanoma cells by ICAM-1 and APN should provide target molecules for new and/or more effective therapeutic approaches.

ICAM-1, as a member of the immunoglobulin superfamily, mediates heterotypical cell cell interactions. The counter receptor of ICAM-1 is the heterodimer integrin LFA-1, expressed by T- and B-lymphocytes, granulocytes and macrophages. ICAM-1 is detectable on advanced human melanomas but not on benign melanocytes or early melanomas. Melanoma cells with a high level of ICAM-1 show an elevated ability to metastasize. In addition, the incidence of ICAM-1 expression correlates with the melanoma thickness. These data suggest that the ICAM-1 expression contributes to the metastatic behaviour of melanoma cells. Mechanisms that are used by ICAM-1-expressing tumor cells comprise the shedding or secretion of soluble ICAM-1 (sICAM-1) that might protect the tumor cell against cytotoxic T-cell attack by blocking the LFA-1 molecules on the T-cell membrane, induction of temporary expression of varying adhesion molecules on endothelial cells (28) as well as tumor cells (29) resulting in optimized cell-cell interactions between tumor and endothelial cells and a reduced adhesion of lymphocytes to endothelial cells and other reactions (5). In contrast to immunohistochemical detection of ICAM-1, sICAM-1 in serum could be a diagnostic or prognostic marker in melanoma patients. However, it was found that besides the secretion from melanoma cells, sICAM-1 is inducible by different cytokines (IL-1, TNFα, IFNγ) and can be derived from different cells (9). Therefore, sICAM-1 serum levels do not necessarily reflect the extent of cell membrane expression specifically on melanomas and may be independently regulated. Indeed, elevated serum levels of sICAM-1 were found not only in patients with different types of tumors, but also in various non-tumor diseases (9). Results of sICAM-1 serum level measurements from patients with different melanoma stages are not consistent. Some data indicate that the serum sICAM-1 concentrations correlate with the progression of melanoma (30) or that the level of sICAM-1 was inversely correlated with the survival of stage II and III patients (31). But Yasasoeve et al. (32) did not find significant differences for sICAM-1 serum levels between patients bearing melanomas of stages I to IV in comparison to the control group. Thus, sICAM-1 is not a specific diagnostic parameter for malignant melanomas, but is at most a prognostic marker if interfering disorders can be excluded. Besides the latter factor, it is notable that sICAM-1 results from melanoma patients arise from the use of different commercial ELISA kits, which are possibly not comparable in sensitivity and precision.

However, cell surface APN could be a target for the detection of malignant melanoma cells and for the reversal of their invasive phenotype. The main properties that define APN as a target molecule in antitumor approaches are its capacity to degrade type-IV collagen (20, 22), to confer invasive ability on tumor cells (invasion through Matrigel) (20, 21), to mediate angiogenesis and to be a receptor for peptides bearing the NGR sequence motif (33). More recent investigations revealed that APN is not the sole target of drugs such as actinonin and bestatin, known as competitive inhibitors of APN (34, 35). Both APN inhibitors were shown to have an antiproliferative effect toward APN-positive human leukemia and lymphoma cells. The growth inhibition of APN-positive tumor cells as well as APN-negative cells (RAJI cells) has shown that the inhibitory effect of actinonin and bestatin cannot be mediated through inhibition of APN alone. In addition, the antiproliferative effect of actinonin on APN-positive cells was not abrogated by pretreatment of cells with the mAb F23, which blocks actinonin binding to the active site in the APN molecule (34). This suggests that the growth inhibitory effect of actinonin on APN-positive tumor cells is not mediated through APN inactivation. Nevertheless, in vitro experiments have shown antiinvasive and antiproteolytic activities using APN inhibitors (actinonin, bestatin, amastatin) as well as APN antibodies in APN-positive melanoma and other tumor cells (20-22). But tumor cell invasion is a complex process which can be mediated by varying factors and there are highly invasive tumor cells which do not express APN.

Substances which inhibit the cellular efflux of drugs have shown an increased antiproliferative activity with actinonin and bestatin in leukemia cells (35). These data suggest that most of the antiproliferative effect of actinonin and bestatin results from their intracellular action. The authors discuss different ways how the inhibitors can enter the cells, including APN-mediated endocytosis. Finally, the intracellular action of actinonin was confirmed by Lee et al. (36). It was found that actinonin and several actinonin analogs inhibit the human peptide deformylase (HPDF), localized in mitochondria, resulting in an antiproliferative effect in 16 human cancer cell lines. Small interfering RNA inhibition of HPDF protein expression was also antiproliferative.

Since growing tumor cells depend on blood vessel formation and APN is involved in the induction of angiogenesis and is up-regulated in both tumor cells and tumor vasculature, tumor-associated endothelial cells are also targets in inhibition experiments, as shown for
curcumin. Curcumin, a phenolic natural product, was shown to be an irreversible inhibitor of APN, producing an antiangiogenic effect (37). Curcumin strongly inhibits APN activity both in vitro and in vivo by directly binding to APN in vitro and in human umbilical vein endothelial cells (HUVECs). The inhibition of APN activity by curcumin resulted in a dose-dependent suppression of tumor invasion of APN-positive tumor cells and of bFGF-induced angiogenic differentiation of HUVECs. Curcumin did not significantly inhibit the invasion of APN-negative tumor cells, suggesting that the antiinvasive activity of curcumin against tumor cells is attributable to the inhibition of APN. This study offers the possibility of targeted inhibition of APN activity in tumor and tumor associated endothelial cells. Although curcumin’s low systemic bioavailability following oral dosing may limit access of sufficient concentrations for pharmacological effect in certain tissues, the attainment of biologically active levels in the gastrointestinal tract has been demonstrated in animals and humans (38).

The other principle for the targeted inhibition of APN-induced angiogenesis in tumor vasculature is realized by introducing an additional NGR sequence into the endostatin molecule (39) or by adding an integrin binding RGD sequence to human endostatin to improve its integrin affinity (40). Endostatin, a C-terminal fragment of collagen XVIII, is described as an endogenous inhibitor of angiogenesis and tumor growth (41). Normal endostatin does not inhibit APN but NGR-endostatin generated an increased binding to and inhibition of endothelial cell APN as well as an elevated inhibition of endothelial cell proliferation and migration (39). While endostatin showed no APN inhibition, NGR-endostatin exhibited 17% and bestatin 35% inhibition. NGR-modified endostatin had a higher antiangiogenic effect than normal endostatin. The growth inhibition of ovarian carcinoma cells in nude mice was 50% by NGR-endostatin and 32.5% by endostatin (39). Although human recombinant endostatin has no toxic effects and generates no drug resistance, its administration in phase I studies was assessed as disappointing (42, 43). Critical commentaries were given to the promise of anti-angiogenic cancer therapy (44) and to the potential and function of endostatin and other matrikines (42, 45). So far ingenious approaches have been realized and quite a few are in progress to produce a therapeutic reversal in tumor growth and metastasis. But the complexity of the outside in signal transduction pathways and their cellular responses is one reason for limited success. More insights into these multifactorial processes and the interrelation between ECM components and their derived matrikines should provide additional methods for a more effective tumor therapy or useful combinations with conventional methods.

References


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