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Procathepsin-L, a Proteinase that Cleaves Human C3 (the Third Component of Complement), Confers High Tumorigenic and Metastatic Properties to Human Melanoma Cells

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Abstract

We previously demonstrated that highly metastatic human melanoma cells secrete a 41 kDa proteinase that cleaves C3, the third component of complement, and shares antigenic determinants with procathepsin-L. Thus, we herein transfected the nonmetastatic DX-3 melanoma cells with the procathepsin-L cDNA. Three clones expressing and secreting high levels of procathepsin-L were selected. Conditioned medium and whole cell extracts from these clones, but not from control cells, carried a high C3-cleaving activity. The transfected clones displayed up to 60% resistance to complement-mediated lysis. Overexpression of procathepsin-L in melanoma cells increased their tumorigenicity and switched their phenotype from nonmetastatic to highly metastatic cells. This is the first report that demonstrates that enforced expression of procathepsin-L by human melanoma cells arms them with the ability to inactivate complement-mediated lysis and contributes to tumor growth and metastasis.

Introduction

Cell surface-associated proteinases that cleave human C3 could protect normal or tumor target cells against autologous complement lysis by preventing deposition of activated C3b on their surface. In addition, proteinases could generate and/or modify C3 fragments, consequently altering their regulatory functions on normal or tumor cells and also modifying immune response because C3 is involved in antigen presentation. This is related to the properties of C3 to act through three distinct mechanisms: (a) C3 plays a pivotal role in complement activation pathways by initiating formation of membrane attack complex on target cell, thus leading to cell lysis (1); (b) activated C3 molecules could interact covalently with antigens, thus enhancing their presentation (2); (c) C3 cleavage generates fragments (such as C3a, C3b, C3bi, C3dg and C3d) that could regulate cellular functions by interacting with specific receptors such as C3aR, C3bR or CR1, C3bi or CR3, C3dg or CR4 and C3d or CR2, respectively (3, 4). Some of us identified three membrane proteinases that cleave C3: p57, p65, and p39/p41. P57 C3-cleaving proteinase is a serine proteinase purified from human erythrocyte membranes (5), identified as a fragment of the 62 kDa spectrin binding domain of ankyrin (6). Using polyclonal anti-p57 Abs we identified a p65 proteinase ex a fragment of the 62 kDa spectrin binding domain of ankyrin (6). Proteinase purified from human erythrocyte membranes (5), identified as p57, p65, and p39/p41. P57 C3-cleaving proteinase is a serine protease that contributes for at least 45% of the resistance of these tumor cells to human complement lysis. Some of us identified three membrane proteinases that cleave C3: p57, p65, and p39/p41. P57 C3-cleaving proteinase is a serine protease that contributes for at least 45% of the resistance of these tumor cells to human complement lysis and was not expressed at the surface of melanoma cell lines susceptible to complement lysis (7). In addition, a murine p39 cysteine proteinase was identified as a new member of the C3-cleaving membrane proteinase family (8). p39 carried amino acid sequence identities with procathepsin-L (8). Inhibition by anti-p39 F(ab')2 fragments of the p39 C3-cleaving proteinase expressed on surface of murine melanoma DSm cells increased up to 60% their susceptibility to complement lysis (8). More recently, we identified the human counterpart of murine p39 procathepsin-L on DM-4 human melanoma cells, which are highly metastatic in nude mice (9). Indeed, these melanoma cells express a C3-cleaving cysteine proteinase activity carried by p41, a proteinase characterized by a Mr 41,000. Precubation of DM-4 cells with anti-p39 F(ab')2 decreased their complement resistance by 45%. In addition, pretreatment of DM-4 cells with anti-p39 Ab strongly inhibited their tumorigenicity and significantly decreased their metastatic potential in nude mice, supporting the notion that the p41 C3-cleaving proteinase contributed to tumorigenicity and metastasis of the DM-4 human melanoma cells (9). Furthermore, these human melanoma cells synthesized and secreted C3, as well as procathepsin-L (10). We herein analyze the protective role of procathepsin-L in tumorigenicity and metastatic potential of human melanoma cells. For this purpose, we transfected human melanoma cells characterized by very low tumorigenic and metastatic properties, with procathepsin-L cDNA and isolated three clones. The transfected cells expressed and secreted a higher level of procathepsin-L protein and C3-cleaving activity, than control cells. In addition, the transfected cells acquired a very high tumorigenic and metastatic properties in nude mice. Overexpression and secretion of procathepsin-L by human melanoma cells resulted in cleavage of C3 and inhibition of complement-mediated lysis, thus contributing to tumor growth and acquisition of the metastatic phenotype.

Materials and Methods

Cells. The human melanoma cell line DX-3, isolated from a cutaneous metastasis from a patient at U. T. M. D. Anderson Cancer Center, is poorly metastatic in nude mice (11). Cells were grown as monolayer, in RPMI 1640 containing 10% FCS, 100 μg/ml streptomycin, 100 units/ml penicillin, 100 μg/ml kanamycin and 2 mM glutamine, at 37°C in a 5% CO2 incubator.

Cell Transfection. Plasmid pGHP3, corresponding to plasmid pGEM3 in which procathepsin-L cDNA was cloned, was kindly provided by Dr. M. M. Gottesman (12). To transfet human cells, procathepsin-L cDNA was subcloned in pCDNA3 plasmid (5.4 kb), using restriction enzymes HindIII and BamHI (Promega). Procathepsin-L cDNA was inserted into the pGHP3 plasmid between PstI and BamHI restriction sites, which are compatible with the ligation sites of the procathepsin-L insert, downstream of T7 promoter. After digestion, pCDNA3 plasmid was purified by electrophoresis on agarose gel and eluted in 30 μl Tris-HCl 10 mM pH 8, 1 mM EDTA (TE) buffer. In parallel, pGHP3 plasmid, digested by HindIII and BamHI restriction enzymes, was also purified by electrophoresis on agarose gel. Procathepsin-L insert (1.5 kb) was eluted in 30 μl TE. Ligation was performed with 30 ng/μl pCDNA3 vector and...
80 ng/µl procathepsin-L insert in the presence of T4 DNA ligase (3 U Weiss/µl) and ligation buffer 10× (300 mM Tris pH 7.8, 100 mM MgCl2, 100 mM DTT, 5 mM ATP). Then, competent DH5α bacteria were transformed. The pcDNA3 plasmid was used to establish stable transfected melanoma cells with procathepsin-L cDNA, as follows: from plasmid DNA prepared in large amount (500 µl TE), a complex of DNA coprecipitated with phosphate (1 ml) was formed and incubated with human melanoma cells. Transfected cells were selected on gentamicin (500 µg/ml). After a week, clones were detected and selected.

Preparation and Analysis of Conditioned Medium. Confluent DX-3 cells before and after transfection were washed with PBS, trypsinized and washed three times by centrifugation at 500 g for 10 min. Then, cells were grown in serum-free RPMI 1640 medium containing 100 µg/ml streptomycin, 100 units/ml penicillin, 100 µg/ml kanamycin and 2 mM glutamine, at 37°C in a 5% CO2 incubator, for 24 h. Conditioned medium was collected by centrifugation, filtered on 0.45 µm (Sartorius) and concentrated 20 times on centriprep 10 (Amicon), with a cutoff at 10 kDa.

Preparation and Analysis of Cell Extracts. Preparation of whole cell extracts in presence of 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate determination of protein concentration using the bicinchoninic acid reagent and analysis of proteins by SDS-PAGE under reducing conditions, as detailed previously (8–10).

C3-cleaving Activity of Cell Samples. Purified C3 was labeled with 125Iodine as described previously (8–10), with a specific activity of 106 cpm/µg. Then, 125I-C3 was incubated with conditioned medium or whole cell extracts, and C3 cleavage was analyzed by SDS-PAGE under reducing conditions, as detailed previously (8–10).

Cytotoxicity Assay. Transfected or nontransfected cells were harvested mechanically by scraping the cells with rubber policeman and washed with complete medium. 51Chromium labeling of cells, sequential cell incubations with monoclonal anti-β2microglobulin Ab (Sigma Chemical Co.) plus rabbit complement, and measurement of radioactivity released in supernatant after complement-mediated lysis were detailed previously (8–10).

Tumor Growth and Experimental Lung Metastasis Assays. To prepare tumor cells for inoculation, cells in exponential growth phase were harvested by brief exposure to 0.25% trypsin-0.02% EDTA solution (w/v). The flask was sharply tapped to dislodge the cells and supplemented medium was added. The cell suspension was pipetted to produce a single-cell suspension. The cells were washed and resuspended in Ca2+ and Mg2+ free HBSS to the desired cell concentration. Cell viability was determined by trypan blue exclusion and only single-cell suspensions of more than 90% viability were used. Subcutaneous tumors were produced by injecting 106 tumor cells/0.2 ml HBSS over the right scapular region. Growth of s.c. tumors was monitored by examination of the mice every day and weekly measurement of tumors with calipers. The mice were killed 35 days after receiving injections, and tumors were processed for H&E staining.

For experimental lung metastasis, 106 tumor cells in 0.2 ml of HBSS were injected into the lateral tail vein (i.v.) of nude mice. The mice were killed after 60 days and the lungs were removed, washed in water, and fixed with Bouin’s solution for 24 h to facilitate counting of tumor nodules, as described previously (13). The number of surface tumor nodules was counted using a dissecting microscope. Sections of the lungs were stained with H&E to confirm that the nodules were melanoma and to monitor the presence of micrometastasis.

Statistical Analysis. The in vivo data were analyzed by the Mann-Whitney test.

Results

Transfection of Human Melanoma Cells with Procathepsin-L cDNA. Previously, we showed that highly metastatic melanoma cells expressed and secreted a 41 kDa C3-cleaving proteinase that shared antigenic determinants with human procathepsin-L. Neutralization of this proteinase led to decrease in complement-mediated lysis and inhibition of tumor growth and metastasis of human melanoma cells in nude mice (9). Here we transfected the nonmetastatic DX3 melanoma cells with the procathepsin-L gene, and subsequently analyzed their ability to cleave human C3 and for changes in their tumor growth and metastasis.

Using expression vector carrying a full length procathepsin-L cDNA, three individual clones were isolated and designated as DX3-cl1, DX3-cl2, and DX3-cl3. The nontransfected DX3 parental and DX3 neotransfected cells (these latter being transfected with pcDNA3 plasmid alone) were used as control.

Secretion of procathepsin-L from transfected cells was first measured in conditioned medium adjusted to the same protein concentration. Samples were submitted to SDS-PAGE, then proteins were transferred on nitrocellulose sheet and immunoblotted using polyclonal anti-cathepsin-L Ab. As shown in Fig. 1, A1, a unique band was identified at 41 kDa, which corresponded to procathepsin-L. Quantification by laser densitometry (Fig. 1, A2), demonstrated that the amount of procathepsin-L is four to five times higher in conditioned medium collected from the three clones (DX3-cl1, DX3-cl2, and DX3-cl3), as compared with conditioned medium from nontransfected cells.

![Fig. 1. Procathepsin-L expression in parental and transfected DX3 melanoma cells.](https://example.com/fig1.png)
DX-3 cells or from DX-3 neotransfected cells. Expression of cathepsin-L forms was also analyzed in 1% NP40 total crude extracts prepared from transfected and control cells. As shown in Fig. 1, B1, anti-cathepsin-L Ab detected a higher amount of cathepsin-L proteins in samples prepared from transfected than from control cells. However, in the transfected clones, cathepsin-L was present in forms characterized by lower apparent Mr 34,000 and Mr 29,000 instead of the procathepsin-L 41-kDa form. Quantification by laser densitometry (Fig. 1, B2), demonstrated that the amount of activated cathepsin-L forms was 10–14 times higher in DX3-cl1, DX3-cl2, and DX3-cl3, than in nontransfected cells.

C3 Cleavage Activity of Transfected Melanoma Cells. We next analyzed the ability of cellular and extracellular forms of cathepsin-L to cleave C3. First, specificity of C3-cleaving activity present in conditioned medium or whole cell extract prepared from each cell line was controlled in the presence of proteinase inhibitors. A typical pattern of C3 cleavage by this proteinase activity, characterized by generation of a 42-kDa fragment, is represented in Fig. 2A. This C3-cleaving activity was inhibited by 1 mM N-(L-3-carboxyl-2,3-trans-carboxyoxirane-2-carbonyl)-L-leucyl-(4-guanidine)-butane, a specific cysteine proteinase inhibitor, but not by 1 mM Pefabloc, a serine proteinase inhibitor. The C3-cleaving activity was observed only in conditioned medium or whole cell extracts prepared from the three clones (DX3-cl1–DX3-cl3) but not from the nontransfected DX3 cells or neotransfected control cells (Fig. 2B). In addition, transfected or nontransfected cells were submitted to complement lysis induced by anti-β2 microglobulin in the presence of complement. These experiments demonstrated that the transfected clones were up to 60% more resistant to complement-mediated lysis than control cells.

Tumorigenic and Metastatic Properties of Procathepsin-L Transfected Melanoma Cells. To determine the tumorigenicity of the procathepsin-L transfected clones which exhibited C3-cleaving activity, we injected 10⁶ cells s.c. into BALB/c nude mice and monitored tumor growth once a week for 35 days. Parental and neotransfected cells grew slowly and did not start to form palpable tumors until 35 days after injection and produced small tumors (0.1 and 0.25 cm in mean diameter). In contrast, the three procathepsin-L transfected clones DX3-cl1, DX3-cl2, and DX3-cl3 grew progressively in all mice that received injections and reached 0.8–1 cm in mean diameter within 35 days (Fig. 3). In the next set of experiments, the metastatic potential of the procathepsin-L transfected clones was determined in an experimental lung metastasis assay (13). To that end, BALB/c nude mice received i.v. injections of 10⁶ DX3 procathepsin-L transfected, parental, and neotransfected cells and, 60 days later, the number of lung metastases was counted. As shown in Table 1, DX3 parental and neotransfected control cells did not metastasize to the lung, whereas the three procathepsin-L transfected clones DX3-cl1, DX3-cl2 and DX3-cl3 produced a high number of lung tumor colonies in all mice that received injections of a median of 156, >200 and >200, respectively (this is representative of two experiments performed). These results demonstrated that transfection of melanoma cells with the procathepsin-L gene increased their tumorigenicity and switched their phenotype from nonmetastatic to highly metastatic cells.
To determine whether the increase in tumor growth and the capacity to produce lung metastases by the procathepsin-L transfected cells in vivo was due to different growth rates in vitro we compared their doubling time to control cells in culture. No difference in doubling time was found between the control and the procathepsin-L transfected clones.

**Discussion**

In a previous report from our laboratories, we demonstrated that highly metastatic melanoma cells expressed and secreted a cysteine proteinase that cleaved human C3 (9). This p41 proteinase shares antigenic determinants with the murine p39 procathepsin-L (8) and human procathepsin-L (9). Moreover, inactivation of p41 activity on melanoma cells by antibodies prepared against murine p39 induced up to 45% decrease in their complement resistance and markedly inhibited their tumorigenic and metastatic potential in nude mice (9).

To analyze the exact contribution of procathepsin-L in these events, we transfected low tumorigenic and nonmetastatic human melanoma cells with procathepsin-L cDNA. Our data demonstrated that only transfected clones, which expressed and secreted a high level of procathepsin-L, also carried a high C3-cleaving activity, identified as a cysteine proteinase associated with the p41 procathepsin-L molecule. Overexpression and secretion of procathepsin-L also rendered melanoma cells resistant to complement-mediated lysis for at least 60%. In addition, transfection of melanoma cells with the procathepsin-L gene increased their tumorigenicity and rendered them highly metastatic in nude mice. Cathepsin-L was suggested to facilitate the invasion and metastasis of tumor cells by degrading components of the basement membrane and extracellular matrix (14). Our study, therefore, provides an alternative and complementary mechanism by which procathepsin-L may contribute to tumor growth and metastasis, i.e., inhibiting melanoma resistance to humoral immune system.

Malignant melanoma cells are resistant to the immune response as evidenced by both their progressive growth in patients despite specific humoral and cellular immune responses to tumor antigens and by the moderate clinical effect of active specific immunotherapy with tumor vaccines tested to date. The present report suggests that this “immune resistance” may be due to the expression of procathepsin-L, which acts as a cell membrane-associated inhibitor of complement component that blocks complement-mediated lysis. This notion is further supported by the observation that the relative specific activity of cathepsin-L was 7-fold greater in subpopulation of “high” metastatic potential of B16 murine melanoma than “low” metastatic potential (14). Enhanced expression of cathepsin-L in metastatic cells is not limited however to malignant melanoma. For example, enhanced cathepsin-L expression was also found in metastatic bone tumors (15). Procathepsin-L was also found to be secreted by human non-small lung cancer cell lines (16) whereas its expression was directly correlated with the progression of human gliomas (17) and colorectal carcinomas (18). In addition, in some patients with ovarian cancer, the levels of cathepsin-L in their serum was elevated (19).

Taken together these observations suggest that expression and secretion of procathepsin-L might be a general mechanism by which tumor cells escape the immune surveillance. As such, procathepsin-L may serve as an excellent marker for the acquisition of the metastatic phenotype by diverse types of tumors (20).

In summary, the data presented herein demonstrated that expression of procathepsin-L by human melanoma cells increased their tumorigenicity and switched their phenotype from nonmetastatic to highly metastatic cells, possibly through its activity to cleave the C3 component of complement, thus, enabling the cells to escape the immune surveillance. Our results may explain the “immune resistance” of human melanoma cells despite being highly immunogenic.

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**References**

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