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Research Article

# Calpains: Markers of tumor aggressiveness?

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ABSTRACT

Rhabdomyosarcoma (RMS) are soft-tissue sarcoma commonly encountered in childhood. RMS cells can acquire invasive behavior and form metastases. The metastatic dissemination implicates many proteases among which are μ-calpain and m-calpain.

Study of calpain expression and activity underline the deregulation of calpain activity in RMS. Analysis of kinetic characteristics of RMS cells, compared to human myoblasts LHCN-M2 cells, shows an important migration velocity in RMS cells. One of the major results of this study is the positive linear correlation between calpain activity and migration velocity presenting calpains as a marker of tumor aggressiveness. The RMS cytoskeleton is disorganized. Specifying the role of μ- and m-calpain using antisense oligonucleotides led to show that both calpains up-regulate α- and β-actin in ARMS cells. Moreover, the invasive behavior of these cells is higher than that of LHCN-M2 cells. However, it is similar to that of non-treated LHCN-M2 cells, when calpains are inhibited. In summary, calpains may be involved in the anarchic adhesion, migration and invasion of RMS. The direct relationship between calpain activity and migration velocities or invasive behavior indicates that calpains could be considered as markers of tumor aggressiveness and as potential targets for limiting development of RMS tumor as well as their metastatic behavior.



Alveolar and embryonal rhabdomyosarcoma (ARMS and ERMS respectively) are soft-tissue sarcoma commonly encountered in childhood and adolescence. These rhabdomyosarcoma (RMS) arise from immature cells which fate is to form striated skeletal muscle. These cells express, similarly to muscle cells, some muscle specific proteins such as myogenic factors (MRF), desmin, and myogenin [1,2]. ARMS are characterized by two chromosomal translocations: t(2;13)(q35;q14) or t(1;13)(p36;q14) [3]. ERMS histological type is associated with a loss of heterozygosis at the 11p15 locus [4]. RMS can acquire invasive behavior and can form metastases in lung, bone, marrow, and lymphatic nodes. The development of such metastases is associated with poor prognosis [5]. The comprehension of mechanisms that regulate cancer cells migration and invasion may be a key for the development of new therapies for limiting metastases. Among the many proteases implicated in cell motility, the calpains play an essential role in regulating migration and invasion phenomena, both involved in metastases development [6–8]. Targeting calpains may present a novel approach toward restraining metastasis and development of RMS cancers. Calpains are calcium-dependent cysteine proteases which best-characterized members are μ-calpain and m-calpain (i.e. CAPN1 and CAPN2 respectively), known as ubiquitous

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heterodimeric enzymes consisting of a large 80-kDa catalytic subunit and a smaller regulatory subunit (about 28 kDa, css1 or css2). Css1 is ubiquitous while css2 is tissue-specific [9].

Calpain activity is $Ca^{2+}$-dependent. Among the different factors regulating calpain activity, the critical factor is its specific endogenous inhibitor, calpastatin [10]. Calpastatin is a ubiquitous protein regulated by several factors [11] that requires $Ca^{2+}$ to bind and inhibits calpains [12].

The present study focuses on the role of both μ- and m-calpain in the metastatic characteristics of the two cancer cell lines, ARMS and ERMS, assuming the role of calpains in cell adhesion and migration (that are limited steps of invasion and metastasis).

To reach that goal, we first characterized the calpain system in RMS before studying the role of the calpains in RMS cell adhesion, on RMS morphology and cytoskeleton organization, as well as in RMS migration characteristics and invasive behavior. Non-tumor human myoblasts LHCN-M2 [13] were chosen as non-metastatic control cells.

The main result is that calpains play a crucial role in adhesion, high migration velocities and invasive behavior of RMS. More specifically, calpains may be considered as markers of tumor aggressiveness since their global activity is linked to migration velocities by a positive linear correlation. Moreover, inhibition of calpain activity in RMS led to migration velocities and invasive behavior similar to those of non-treated LHCN-M2 cells. Because calpain activity is directly involved in migration and invasion of RMS, the proteases would be a good target for the development of novel therapies to control metastasis.

## Materials and methods

### *Cell culture*

Cells were grown under 5% $CO_2$ atmosphere at 37°C in DMEM (Dulbecco's modified Eagle medium) with pyruvate supplemented with 10% FBS (fetal bovine serum) for RMS, and a culture medium containing DMEM without pyruvate (60%), medium 199 (20%) and FBS (20%) for LHCN-M2 cells (human myoblasts cell lines) [13]. All cell culture reagents were purchased from Gibco-BRL. Some experiments have been done only with the tumor cell line presenting the most aggressive phenotype, ARMS.

### *Calpain inhibitor treatments*

The chemical calpain inhibitor calpeptin (Z-Leu-Nle-CHO) was purchased from Calbiochem and used at 40 and 60 μM for adhesion assay, 50 μM for migration and invasion assay for immunolocalization and for cytoskeleton organization and 80 μM for Western blot. A second calpain inhibitor, calpain inhibitor III (Z-Val-Phe-CHO, Calbiochem) was used at 50 μM. Antisense oligodeoxynucleotide transient transfections has been realized as previously described [14].

### *Quantification of calpain activity*

All cell lines were cultured in DMEM supplemented with 0.1% FBS during 48 h. The global calpain activity was observed and quantified on living cells using *t*-BOC-LM-CMAC (7-amino-4-chloromethylcoumarin, *t*-BOC-L-leucyl-L-methionine amide, Molecular Probes) [7,15]. This molecule enters passively in cells and becomes fluorescent after calpain cleavage. Culture medium was replaced by DMEM without serum containing 50 μM of *t*-BOC-LM-CMAC. Cells were incubated during 30 min in darkness. The μ-calpain activity was observed and quantified on fixed and permeabilized cells using Calpain 1 substrate (Chemicon) which become fluorescent after μ-calpain cleavage. After PAF fixation (15 min) and permeabilization with 1% Triton X-100 (3 min), cells were incubated with Calpain 1 substrate 50 μM during 40 min in darkness. The global calpain or the μ-calpain activities were then observed using an inverted epifluorescence microscope (Leica AF DMI6000). The results were quantified using the Metamorph software (Molecular Device).

### *Immunolocalization of calpains and calpastatin*

All cell lines were cultured in DMEM supplemented with 0.1% FBS for 48 h. Next, cells were fixed and permeabilized. A specificity was disrupted by incubation (1 h) with PBS/BSA (3%). Then, m-calpain, μ-calpain and calpastatin were detected using corresponding antibodies (N-19 sc-7533 raised in goat (1/50), Santa Cruz Biotechnology; MAB3104, raised in mouse (1/500), Chemicon; H-300 sc-20779, raised in rabbit (1/50), Santa Cruz Biotechnology respectively, for 3 h). Then, cells were incubated with an appropriate secondary Alexa fluor antibody (anti-goat-Alexa594-conjugated (1/1000), anti-mouse-Alexa594-conjugated (1/3000), and anti-rabbit-Alexa488-conjugated (1/1000) antibodies respectively for 90 min). Next cells were observed using an epifluorescence microscope (Leica AF DMI6000).

### *Adhesion assay*

Adhesion assays were performed as described previously [14]. These assays were carried out with (40 μM or 60 μM) or without calpeptin treatment.

### *Migration assay*

All cell lines were seeded in DMEM containing 0.1% FBS at a density of $4.10^3$ cells/$cm^2$ in 35 mm diameter glass-bottom plate. Eight hours after seeding, time-lapse microscopy experiments were performed on an inverted Leica AF DMI6000 microscope equipped with an environmental chamber with phase-contrast optic (images taken every 15 min). Cell velocities of migration, defined as the average of 73 subsequent cell centroid displacements/one time interval between two successive images (15 min), were evaluated using the tracking object of the Metamorph software (Molecular Device). The cell trajectories were recorded for 18 h. These assays have been made in presence vs. absence of calpeptin (50 μM) added at the beginning of the time-lapse.

### *Invasion assay*

The invasive capacity of RMS and LHCN-M2 cells was measured using BD Biocoat Growth Factor Reduced Matrigel Invasion Chambers (BD Biosciences). Cells were seeded at a density of $10^5$ cells/$cm^2$ on the membrane in DMEM with 0.1% FBS. Quiescent medium was also added under the membrane. After 48 h of incubation, cells staying on the membrane were scraped off. Cells lying under the membrane were fixed with PAF (4%), and