Cannibalism of Live Lymphocytes by Human Metastatic but Not Primary Melanoma Cells

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Abstract

The phenomenon of cell cannibalism, which generally refers to the engulfment of cells within other cells, was described in malignant tumors, but its biological significance is still largely unknown. In the present study, we investigated the occurrence, the *in vivo* relevance, and the underlying mechanisms of cannibalism in human melanoma. As first evidence, we observed that tumor cannibalism was clearly detectable *in vivo* in metastatic lesions of melanoma and often involved T cells, which could be found in a degraded state within tumor cells. Then, *in vitro* experiments confirmed that cannibalism of T cells was a property of metastatic melanoma cells but not of primary melanoma cells. In particular, morphologic analyses, including time-lapse cinematography and electron microscopy, revealed a sequence of events, in which metastatic melanoma cells were able to engulf and digest live autologous melanoma-specific $CD8^+$ T cells. Importantly, this cannibalistic activity significantly increased metastatic melanoma cell survival, particularly under starvation condition, supporting the evidence that tumor cells may use the eating of live lymphocytes as a way to "feed" in condition of low nutrient supply. The mechanism underlying cannibalism involved a complex framework, including lysosomal protease cathepsin B activity, caveolae formation, and ezrin cytoskeleton integrity and function. In conclusion, our study shows that human metastatic melanoma cells may eat live T cells, which are instead programmed to kill them, suggesting a novel mechanism of tumor immune escape. Moreover, our data suggest that cannibalism may represent a sort of "feeding" activity aimed at sustaining survival and progression of malignant tumor cells in an unfavorable microenvironment. (Cancer Res 2006; 66(7): 3629-38)

Introduction

Cannibalism is recognized as a phenomenon commonly used by unicellular and higher organisms, even at single-cell level, as a survival option. We did not know whether cells, able to feed through other cells, were present in a normal human body, but cannibal cells have been identified in malignant tumors up to a century ago (1, 2). More recently, cells with cannibalistic behavior have been detected in tumors of different histology (3–6), and their presence was related to a poor prognosis (3, 7). Cannibal tumor cells have been called in different ways, such as "bird-eye cells" or "signet-ring cells," but the significance of tumor cannibalism and the mechanism(s) underlying this phenomenon are still unknown. Particularly, there is no agreement on the nature of the cannibal cells and the nature of the cells contained within them (8). For instance, it is not clear whether cannibal cells are the result of an aberrant cell division or a phagocytic process and whether the cells contained within the cannibal cells are dead or alive. The general notion was that cannibal cells contained other tumor cells and, therefore, that tumor cells fed among themselves. However, some reports suggested that tumor cell cannibalism may involve engulfment of neutrophils and erythrocytes (7, 9), implying that cannibal tumor cells do not distinguish or select between the normal (including stromal or tumor-infiltrating immune cells) and sibling neoplastic cells.

We have shown previously that metastatic melanoma cells but not primary melanoma cells, compared with primary macrophages, were able to phagocytose apoptotic cells or plastic beads (10). This evidence suggested that a macrophage-like activity could be a unique property of metastatic melanoma cells. A general phagocytic activity may recall the concept of cannibalism (3–7). Thus, we hypothesized that the phagocytic activity of melanoma cells could involve the ingestion of live cells. In turn, the aim of the present study was to investigate the existence and the relevance of tumor cannibalism in human melanoma and to identify the mechanisms underlying this phenomenon.

Materials and Methods

Cell Cultures

The autologous cell system used was represented by the HLA-A*0201$^+$ human melanoma cell line 501mel derived from a metastatic tumor lesion and the autologous MART-1/Melan-A_{27-35}-reactive HLA-A*0201-restricted $CD8^+$ cytotoxic T-cell clone A42 (11). Other human melanoma cell lines used were (*a*) metastatic melanomas: Me30631, Me26258, Me14783, Me10249, Me17781, Me624.38, Me15392, Me8621 (obtained from melanomas of patients surgically resected at the Istituto Nazionale dei Tumori, Milan, Italy), Me116, and Me160 (kindly provided by Dr. Michele Maio, Centro di Riferimento Oncologico, Aviano, Italy) and (*b*) primary melanomas: Me5810P, Me9923P, Me10538P, Me1007P, Me20842P, Me4405P (obtained from melanomas of patients surgically resected at the Istituto Nazionale dei Tumori), WM904, and WM743 (a gift from Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA). Metastatic and primary melanoma cell lines were followed by a progressive number.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

L. Lugini and P. Matarrese contributed equally to this work. L. Rivoltini, W. Malorni, and S. Fais are the principal investigators.

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Human macrophages were obtained after separation of peripheral blood mononuclear cell by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient and then by 46% Percoll (Biochrom KG, Berlin, Germany) density gradient of buffy coats from healthy donors; monocytes were left to differentiate for 1 week at 37°C in RPMI 1640 plus 15% FCS.

All melanoma cell lines as well as the NIH-3T3 cells (Swiss mouse embryonic fibroblast cell line) and macrophages were seeded in 3-cm Petri dishes (2×10^5 or 4×10^5 per dish) in RPMI 1640 supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin (Life Technologies, Gaithersburg, MD), and 2 mmol/L glutamine (Life Technologies) with 10% FCS in a 5% CO_2 environment at 37°C. Melanoma cells were also seeded in condition of serum starvation for a period of 24, 48, or 72 hours.

Immunocytochemistry and Immunohistochemistry

Tumor samples were obtained from surgical specimens of 30 melanoma patients (20 from metastatic lesions and 10 from the primary tumor), embedded in optical cutting temperature compound medium (Tissue-Tek, Bayer, Zurich, Switzerland), and snap frozen. Air-dried acetone-fixed cryostat sections were immunostained by polyclonal antibodies to CD45 or CD3 (Dako, Glostrup, Denmark) using the peroxidase anti-peroxidase method (Dako) in single staining as described previously (12). For each metastatic lesion, two to five areas were picked out, and 300 tumor cells per area were counted to determine the percentages of tumor cells containing $CD3^+$ T cells. Images have been collected at the same ×10 magnification, elaborated by a camera scanner for universal light microscopy application (AxioCam, Carl Zeiss Vision, Oberkochen, Germany), and electronically elaborated through a modular image processing and analysis system (AxioVision, Carl Zeiss Vision), allowing electronic magnification of the acquired images and measurements of any recognizable cellular structures (13).

For immunocytochemistry, human melanoma cell lines, incubated at the melanoma cell to lymphocyte ratio of 1:2.5 on glass chamber slides (LabTek, Naperville, IL), were fixed with 80% methanol for 10 minutes at 4°C and stained with the monoclonal antibody (mAb) to MART-1/Melan-A (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom) or with a polyclonal antibody to CD8 (Dako) using the peroxidase anti-peroxidase method in single staining (12).

Microscopy

Time-lapse cinematography was obtained with the use of a phase-contrast Nikon (Melville, NY) inverted microscope equipped with a Zeiss charge-coupled device camera and a JVC (Pinebrook, NY) time-lapse videotape recorder. Human melanoma cell lines were incubated at the ratio of 1:2.5 with live autologous lymphocytes. Films were recorded under standard culture conditions.

For transmission electron microscopy (TEM) examination, melanoma cell lines in monolayer were incubated at the ratio of 1:2.5 with autologous lymphocytes and then fixed in 2.5% glutaraldehyde and 1% OsO_4 in cacodylate buffer (0.2 mol/L; pH 7.2). After dehydration through graded ethanol, monolayer was *in situ* embedded in epoxy resin (Agar Aids, Cambridge, United Kingdom). Ultrathin sections (70 nm) were counter-stained with uranyl acetate and lead citrate and observed with a Philips 208 electron microscope (FEI Company, Hillsboro, OR).

For scanning electron microscopy (SEM) examination, cells were fixed as described above, dehydrated in ethanol, critical point dried in CO_2, and gold coated by sputtering. The samples were examined with a Cambridge 360 SEM.

Phagocytic Assay of Live Lymphocytes by Tumor Cells

Twenty-four hours after seeding, melanoma cell lines, either untransfected or transfected with small interfering RNA (siRNA) to cathepsin B, were incubated with different ratios (1:10, 1:5, and 1:2.5) of autologous live lymphocytes, stained with 10 μmol/L dihydrorhodamine 123 (DHR123; Molecular Probes, Leiden, the Netherlands) or 5 μmol/L hydroethidine [HE; for green fluorescent protein (GFP)–transfected clones; Molecular Probes], and incubated at 37°C for different times (1.5, 3, 5, or 18 hours). After washings to remove noninternalized lymphocytes, melanoma cells were harvested and analyzed on a cytometer equipped with a 488-nm argon laser. Melanoma cells that appeared fluorescent in green (if lymphocytes were stained with DHR123) or red (if lymphocytes were stained with HE) were considered as phagocytic. To discriminate between lymphocytes, internalized or adherent to the melanoma cell surface, the phagocytic assay was also done by either pretreating with 0.2 μg/mL cytochalasin B (Sigma-Aldrich, Milan, Italy) or incubating cells at 4°C. As negative control, macrophages and NIH-3T3 fibroblast were used in the same conditions. All the samples were recorded with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with a 488-nm argon laser. At least 20,000 events have been acquired and statistically analyzed by a Macintosh computer using CellQuest software (Becton Dickinson).

Transfection of Melanoma Cells

Ezrin mutant. The GFP-tagged deletion mutant of ezrin (patent 01318913.1) was obtained as a GFP-mutant ezrin fusion protein, in which a 147-585 were deleted. Primers used were the following: 5′-CTGCAGACT-CACCAGAAACCGA-3′ and 5′-GGTACCCAGACTTGTGCACTTC-3′. PCR product was cloned into pTopo vector (Invitrogen, Milan, Italy), then excised with *Xho*I and *Eco*RI restriction enzymes (Promega, Milan, Italy), and ligated in the pEGFP-N1 vector (Clontech Laboratories, Inc., Mountain View, CA) to produce the fusion protein. Plasmid encoding the GFP-mutant ezrin fusion protein was transfected into melanoma cells using Lipofect-AMINE 2000 transfection kit (Invitrogen). Percentage of transfected cells was evaluated by fluorescence-activated cell sorting (FACS) analysis. No alterations in terms of morphologic features, proliferation, and cell cycle progression were observed among the GFP-mutant ezrin-transfected clones compared with wild-type (WT) cell lines.

Cathepsin B gene silencing with siRNA. The siRNA targeting human cathepsin B were the following: r(GGAUCACUGCGGAAUCGAA)dTdT and r(UUCGAUUCCGCAGUGAUCC)dTdG. siRNA was synthesized by Qiagen (Hilden, Germany) and annealed according to the manufacturer's instructions. Melanoma cells were transfected using HiPerFect transfection reagent (Qiagen) according to the manufacturer's instructions. Briefly the day before transfection, melanoma cells were seeded in six-well plates (1×10^5 per well), and after 24 hours, cells were transfected with 5 nmol/L siRNA per well. Forty-eight hours after transfection, cells were analyzed for cathepsin B expression by FACS analysis as described above.

Evaluation of Lysosomal Acidity

Human melanoma cells were stained with 1 μmol/L LysoSensor probe (Molecular Probes) for 5 minutes at 37°C and immediately analyzed by a cytometer. Comparisons among different melanoma cell lines were conducted by CellQuest software using the median values of fluorescence intensity histograms. Statistical analysis was done by the parametric Kolmogorov-Smirnov test on a population of at least 20,000 cells. Only Ps < 0.01 were considered as statistically significant.

Survival in Acidic Microenvironment

Cell lines were seeded in RPMI 1640 with 10% FCS at different pH values: 7.2, 6.0, and 5.0. These were obtained by adding 2 N HCl to the RPMI 1640 culture. After 2 days, cells were stained with 0.4% trypan blue and analyzed by flow cytometry.

Expression Levels of Cathepsin B and D in Melanoma Cells and Treatments with their Inhibitors

Human melanoma cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 (Sigma-Aldrich), and stained with a specific polyclonal antibody to cathepsin B or D (Calbiochem Co., Darmstadt, Germany) and then with an anti-rabbit IgG FITC-conjugated antibody (Sigma-Aldrich). Samples were analyzed with a FACScan flow cytometer. To further evaluate the role of cathepsin B or D in phagocytic activity, metastatic melanoma cell lines were treated with (*a*) CA-074 Me (10 μmol/L; Calbiochem), a specific inhibitor of the cysteinyl protease cathepsin B, or (*b*) pepstatin A (100 μmol/L; Sigma-Aldrich), a specific inhibitor of the aspartyl protease cathepsin D, 2 hours before phagocytic test.

Involvement of Caveolae in Phagocytic Activity of Metastatic Melanoma Cells

The internalization of live lymphocyte was also investigated treating metastatic melanoma cell lines with different concentrations (1, 3, 5, or 10 μg/mL) of filipin (Sigma-Aldrich) for 1 hour before phagocytic assay.

Survival Evaluation of Melanoma Cells after Lymphocyte and Latex Bead Ingestion

Melanoma cells were incubated for long time (18 hours; to maximize their phagocytic activity) at 37°C with or without live lymphocytes or latex beads and analyzed for cannibalistic activity by flow cytometry as described above in the standard RPMI 1640 with 10% FCS, without FCS, and in HBSS. After 5 days, melanoma cells were further washed, harvested, incubated for 15 minutes at 37°C with propidium iodide (40 μmol/L), and immediately analyzed by flow cytometry. Lymphocytes or latex beads were distinguished from melanoma cells by analyzing the physical variables (forward and side scatter).

Immunoprecipitation and Western Blot

Subconfluent melanoma cells were lysed in AKT buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1% NP40] with protease inhibitors (10 μg/mL aprotinin and 2 mmol/L phenylmethylsulfonyl fluoride) or were subjected to differential ultracentrifugation as described previously (14) to obtain a pellet enriched in endolysosomes. Endolysosomal pellet was resuspended in immunoprecipitation buffer [50 mmol/L HEPES (pH 6.9), 10 mmol/L EDTA, 1% Triton X-100, 300 mmol/L NaCl]. Proteins were precleared with protein A/G-Sepharose 4B Fast Flow (Sigma-Aldrich). Ezrin or caveolin-1 proteins were immunoprecipitated from precleared lysates with either anti-ezrin mAb (Sigma-Aldrich) or anti-caveolin-1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in the presence of protein A/G-Sepharose, resolved in 12% SDS-PAGE, and electroblotted on nitrocellulose (Protran BA85; Schleicher & Schuell, Keene, NH). Blotting was done using polyclonal antibody to caveolin-1 and mAb to lysosomal-associated membrane protein-1 (LAMP-1; PharMingen, San Diego, CA), ezrin (Sigma-Aldrich), and actin (Chemicon International, Inc., Temecula, CA). After incubation with appropriate peroxidase-conjugated anti IgG (Amersham Biosciences, Milan, Italy), membranes were revealed by enhanced chemiluminescence (Pierce, Rockford, IL).

Cytotoxic Assay

Cytotoxic activity of the anti-MART-1/Melan-A $CD8^+$ T-cell clone A42 (15) was evaluated by a standard 4-hour ^{51}Cr release assay. As target cells, the autologous melanoma line (derived from a metastatic lesion) 501mel was used. One thousand ^{51}Cr-labeled target cells per well were coseeded for 4 hours in V-bottomed 96-well plates with the effector cells at different E:T ratios. Spontaneous target lysis was evaluated by incubating target cells with medium, whereas total target lysis was determined by treatment with 2% NP40 detergent-supplemented medium. The percentage of specific lysis was calculated as follows: [(experimental cpm − spontaneous cpm) / (total cpm − spontaneous cpm)] × 100. The assay was done in triplicate.

Phagocytosis of Anti-MART-1–Specific $CD8^+$ T Cells by WT or Mutant Ezrin Melanoma Cells and Cytotoxicity Evaluation

501mel cell lines (WT or ezrin mutant) were stained with PKH26 red fluorescent cell linker (Sigma-Aldrich) according to the manufacturer's instructions, whereas the anti-MART-1 $CD8^+$ T-cell clone A42 (11) was labeled with an anti-CD8-APC mAb (PharMingen). Cells were then cocultured for 1 hour at 37°C at different lymphocyte to tumor ratios in round-bottomed 96-well plate, harvested, washed with PBS supplemented with 1% FCS, and analyzed using FACSCalibur (Becton Dickinson) and CellQuest software. Data are PKH26 tumor cells staining positive for CD8-APC (numbers indicate percentage of $PKH26^+/CD8^+$ cells).

Data Analysis and Statistics

All samples were analyzed with a FACScan cytometer equipped with a 488-nm argon laser. At least 20,000 events were acquired. Data were recorded and statistically analyzed by a Macintosh computer using CellQuest software. The expression level of the analyzed proteins was expressed as a median value of the fluorescence emission curve, and the statistical significance was calculated by using the parametric Kolmogorov-Smirnov test. Statistical analysis of phagocytic and survival data was done by using Student's *t* test or one-way ANOVA by using StatView program for Macintosh. All data reported were verified at least in four independent experiments and expressed as mean ± SD. Only Ps < 0.01 were considered as statistically significant.

Results

Cannibal cells containing T cells are detectable in metastatic tumors. To investigate the relevance of cannibalism *in vivo*, we randomly selected histologic samples of melanoma metastatic lesions derived from 20 different stage IV patients and searched for melanoma cells containing cells of different origins. We first analyzed by immunostaining for leukocytes ($CD45^+$ cells) and T cells ($CD3^+$) the possibility that melanoma cells may contain cells of immune origin. We observed that typical tumor cannibalism involved 10% to 12% of cells. These cannibal cells were $CD45^-$ and $CD3^-$ with a mean diameter of 10 to 20 μm, suggesting that they were sibling tumor cells (data not shown). However, in the same sections, some tumor cells containing $CD45^+$ cells, very close to the leukocyte infiltrate, were also detectable (Fig. 1*A*). In all samples, T-cell infiltrates seemed mostly confined at the periphery of the metastatic lesion, with few T cells scattered within the tumor tissue. In these areas, some melanoma cells contained apparently intact $CD3^+$ T lymphocytes (Fig. 1*B*), which in turn seemed to undergo degenerative phenomena within the cannibal cells (Fig. 1*C*). Tumor cells containing $CD3^+$ cells were detectable in 100% of the examined metastatic tissues. The cannibal cells containing $CD3^+$ cells ranged from 2% to 5% (mean ± SD, 3.3 ± 2.1). The majority of these cells were characterized by large, confluent cytoplasmic vacuoles that contained $CD3^+$ cells. However, many other melanoma cells had in their cytoplasm vacuoles apparently empty or containing only cell remnants (Fig. 1*C*). Nevertheless, counts were based exclusively on those cells with a crescent-shaped nucleus and with vacuoles containing cells clearly expressing CD3. Interestingly, in the primary melanoma tissues examined (10 surgical specimens from 10 individual patients), figures of cannibalism toward sibling tumor cells were very rare and tumor cells containing T lymphocytes were undetectable (data not shown).

This set of *in vivo* observation suggested that tumor-infiltrating leukocytes, including T lymphocytes, could represent a target of melanoma cell cannibalism. The engulfed T cells seemed to undergo digestion within the cannibal melanoma cells.

Morphologic features of cannibal melanoma cells. To further investigate this *in vivo* observation, we used an *in vitro* model, analyzing melanoma cell lines and autologous melanoma-specific $CD8^+$ T lymphocytes (11). We chose to use this model to resemble as much as possible the *in vivo* situation of a tumor-specific T cell infiltrating a melanoma lesion.

As a first step, we did a series of immunocytochemical analyses (using MART-1 as specific marker) of melanoma cell cultures, which showed the presence of typical figures of cannibalism as represented by "a tumor cell in the cytoplasm of a larger tumor cell with a crescent-shaped nuclei" (ref. 3; Fig. 1*D*) as well as other tumor cells undergoing cell death (Fig. 1*E*). The cannibal cells were exclusively detected in the cell lines deriving from metastatic melanomas, and the percentage of cannibal cells in the metastatic melanoma cell lines ranged from 15% to 30% (mean ± SD, 23 ± 7). This evidence was consistent with previous reports on cell lines deriving from tumor of different origins (3–6).

Subsequently, we carried out the same type of analysis after coculture of the metastatic melanoma cell line 501mel (representative of metastatic melanomas with phagocytic activity) with the autologous tumor-specific $CD8^+$ T-cell clone A42 (recognizing the melanoma antigen MART-1/Melan-A in a HLA-A*0201-restricted fashion). These experiments showed that, following

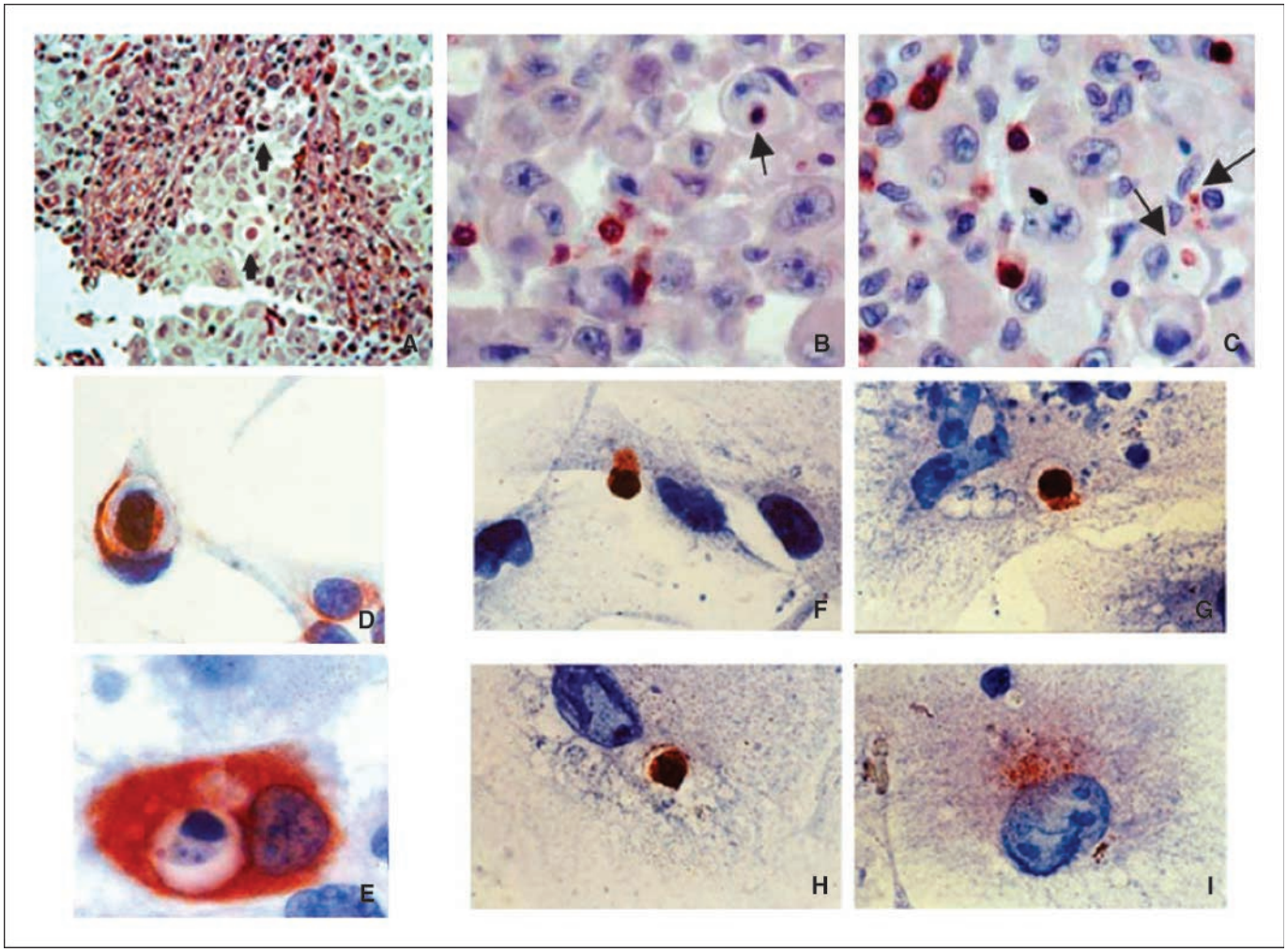

Figure 1. *In vivo* and *in vitro* occurrence of cannibalism in human metastatic melanoma cells. *A,* immunohistochemistry analysis of a cryostat section of a human melanoma showing a leukocyte infiltrate (anti-CD45 staining). *Arrows,* cannibalism. *B* and *C,* immunohistochemistry analysis of a cryostat section of a human melanoma showing a $CD3^+$ T-cell infiltrate within a human melanoma sample. *Arrows,* $CD3^+$ T cell within a melanoma cell in (*B*) and some remnants of $CD3^+$ material within the melanoma cell (*C*). *D,* typical cannibalism in cultured melanoma cells stained for MART-1. *E,* cannibal cell containing a melanoma cell undergoing death in the same melanoma cell cultures. *F* to *I,* possible *in vitro* sequence of melanoma cannibalism, starting from early adhesion, internalization, and final degradation of the ingested autologous $CD8^+$ T lymphocytes.

an early contact (Fig. 1*F* and *G*), T cells (identified by CD8 expression) could be found inside melanoma cells, where they underwent a progressive degradative process (Fig. 1*H* and *I*). By this technique, 12% to 24% melanoma cells contained $CD8^+$ T cells after 2-hour coculture with the A42 T-cell clone. Similar results were obtained using resting $CD8^+$ or $CD4^+$ T cells from healthy donors (data not shown), suggesting that cannibalism by melanoma cells was an unrestricted phenomenon that did not exclusively target antigen-specific T cells. The data obtained by immunocytochemistry were confirmed by time-lapse cinematography, showing a real-time evaluation of the process of live lymphocyte ingestion by metastatic melanoma cells. This analysis showed that T cells, after an early contact with the melanoma cells, suddenly disappeared under its surface (Fig. 2*A-D*; Supplementary Movie).

We then compared in the same *in vitro* model (i.e., 501mel with A42 T-cell clone) cell surface events (by SEM) and the subsequent intracellular events (by TEM) characterizing the cannibal process. The results, obtained using melanoma cell monolayers, showed that live lymphocytes were internalized by melanoma cells through a sequence of events, including an early intimate interaction (a sort of "fusion-like" process; Fig. 2*E* and *F*), tumor cell invagination (Fig. 2*G* and *H*), and entrapment of live lymphocyte within melanoma cells (Fig. 2*I* and *J*). This phenomenon seemed to significantly differ from the typical phagocytosis, where the engulfment is instead preceded by the formation of extended ruffles and pseudopods embracing and engulfing the external body (16).

Cannibalistic activity is a selective feature of metastatic melanoma cells. Using immunocytochemical analysis of melanoma cell monolayers cocultured with the melanoma-specific $CD8^+$ T-cell clone A42 or other $CD8^+$ T cells, we observed that melanoma cells derived from primary lesions did not display any cannibalistic activity toward T cells (data not shown), consistent with the inability of primary melanoma cells to phagocytose apoptotic cells and plastic beads (10).

We then set up a new method allowing us to quantify the internalization by melanoma cells of fluorescent live A42

T lymphocytes stained by DHR123. The results of Fig. 3*A* showed that macrophages and melanoma cells derived from five primary tumor cell lines did not contain fluorescent live lymphocytes, whereas all the five tested metastatic melanoma cell lines were able to cannibalize live lymphocytes. Cannibalism of live T lymphocytes was increased in conditions of serum starvation in metastatic melanoma but not in primary melanoma or macrophage cells (Fig. 3*B*), further supporting the hypothesis that cannibalism, toward either sibling cells or T lymphocytes, may be a specific feature of metastatic melanoma cells.

Cannibalism toward T cells as survival factor. To test whether the ingestion of lymphocytes could confer to metastatic melanoma cells a significantly increased survival in the absence of nutrient supply, we analyzed viability of melanoma cells in conditions of serum and amino acid starvation in the presence or absence of live T lymphocytes. The results showed a clear increase in the percentage of surviving cells when melanoma cells were cultured in the presence of live lymphocytes (Fig. 4*A*), whereas the addition of latex beads did not show detectable effect on tumor cell survival (Fig. 4*A*). In the same set of experiments, primary melanoma cells did not show to phagocyte neither live lymphocytes nor latex beads without any detectable modification of the melanoma cell viability (data not shown). In a separate set of experiments, we also showed that metastatic melanoma cells were able to survive at low pH (5.0), whereas primary melanoma cell lines rapidly died when cultured under the same conditions (Fig. 4*B*). These last data supported the evidence that malignant tumors grow and expand in an acidic environment created by the hypoxic condition (17) and that

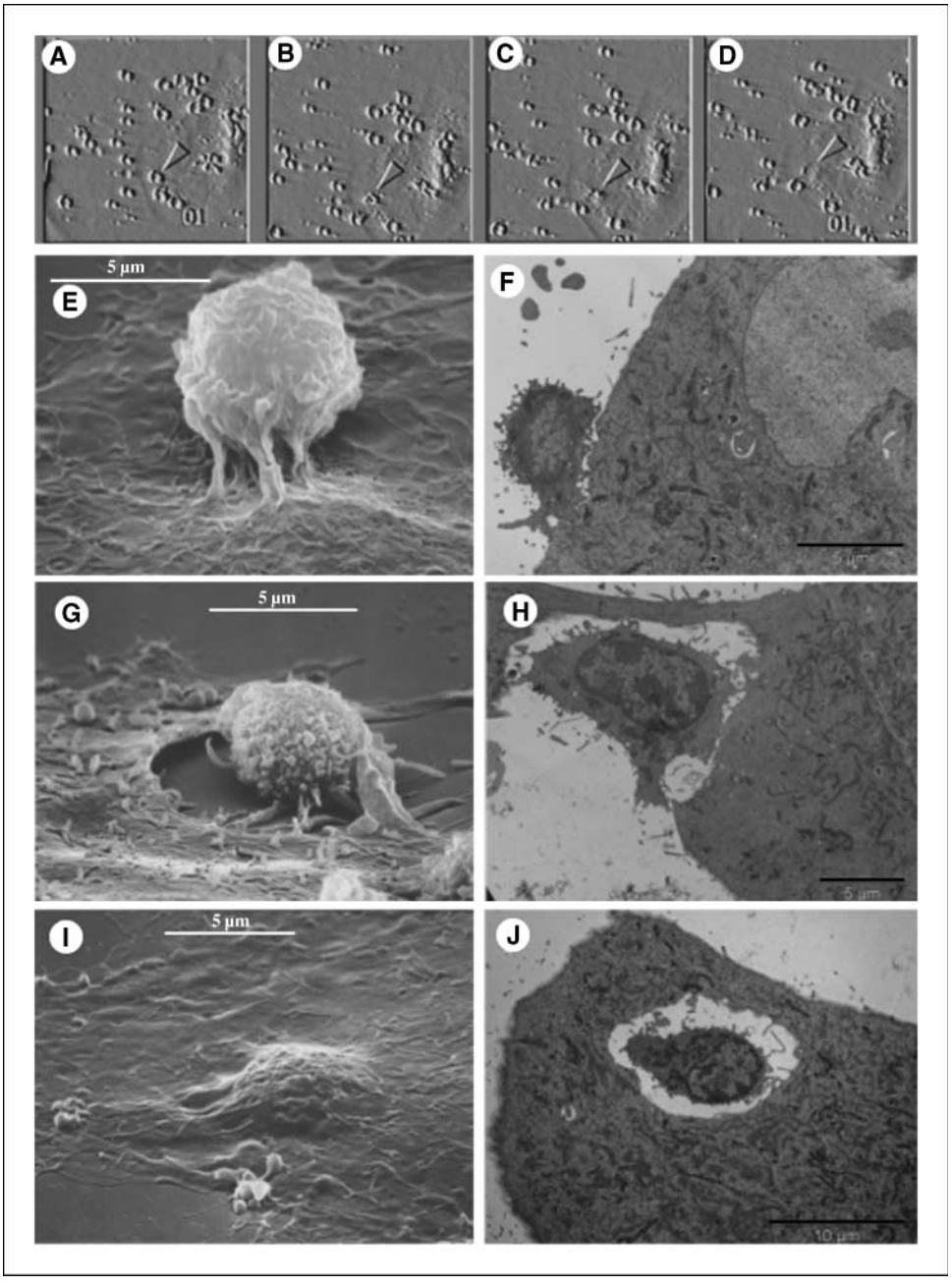

Figure 2. Morphologic analyses of lymphocyte-melanoma cell interaction in cannibalism. *A* to *D,* time-lapse cinematography analysis (see Supplementary Movie) of a coculture of live autologous $CD8^+$ T lymphocytes and metastatic melanoma cells (melanoma cell to lymphocyte ratio of 1:2.5). SEM (*E, G*, and *I*) and TEM (*F, H*, and *J*) of a coculture of live autologous $CD8^+$ T lymphocytes and metastatic melanoma cell monolayer (melanoma cell to lymphocyte ratio of 1:2.5). *E* and *F,* intimate contact between a lymphocyte and a melanoma cell. *G* and *H,* lymphocyte embraced by melanoma cells. *I* and *J,* internalized lymphocyte.

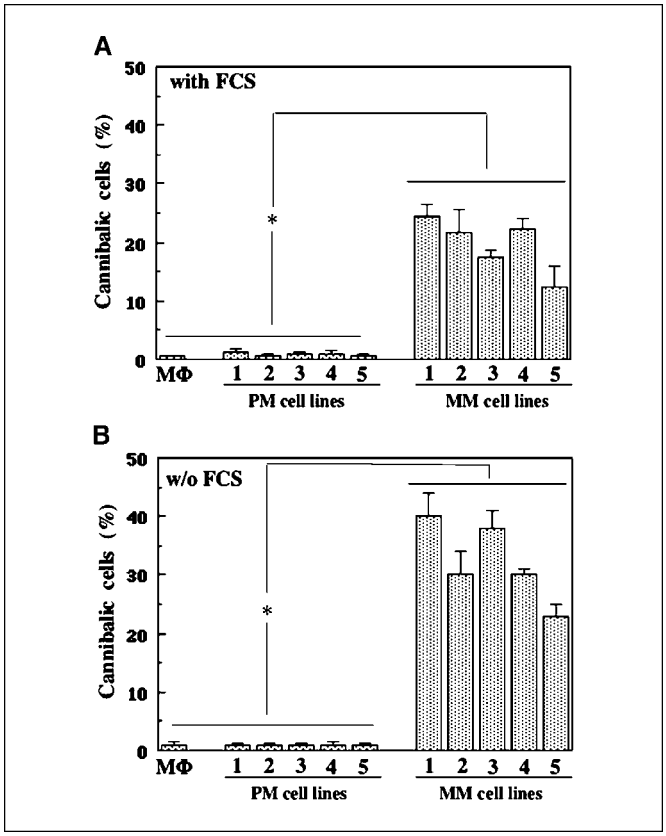

Figure 3. Cannibalistic activity of metastatic melanoma cells. Quantitative evaluation of cannibalistic activity by flow cytometry. Macrophages (*MΦ*), five primary melanoma cell lines (*PM1-5*), or metastatic melanoma cell lines (*MM1-5*) were incubated 3 hours with DHR123-stained lymphocytes in the presence (*A*) or absence (*B*) of FCS. *Columns,* mean of at least four independent experiments done in triplicate; *bars,* SD. *, $P < 0.01$.

treatment with anti-acid drugs inhibits the acidic vesicle-mediated tumor resistance to cytotoxic drugs (18).

Mechanisms involved in the cannibalistic activity of melanoma cells. We first examined the level of intracellular acidity and the digestive enzymes in cells deriving from metastatic lesions compared with cells deriving from primary melanomas. The results showed that the pH of intracytoplasmic vesicles was significantly more acidic in metastatic melanoma cells with respect to primary melanoma cells (Fig. 5*A*), suggesting that the pH value in the acidic organelles of melanoma cells was directly related to their cannibalistic activity against the live cells. These data were consistent with the evidence that the majority of metastatic cell lines expressed high levels of vacuolar ATPases (18), which are involved in the acidification of lysosomal-like vesicles through the proton transport across the membranes at the expense of ATP (18) and directly related to the phagocytosing activity of the cells (19).

Then, we analyzed in melanoma cells the expression of proteolytic enzymes, including a set of proteases known to be activated in acidic conditions, such as cathepsin B and D (20). As revealed by flow cytometry analysis, metastatic melanoma cells displayed significantly ($P < 0.01$) higher levels of the cysteine endopeptidase cathepsin B compared with primary melanoma cells (Fig. 5*A*). By contrast, the aspartyl protease cathepsin D was equally expressed in both primary and metastatic melanomas (Fig. 5*A*), supporting previous evidence on the specific role of cathepsin B in the acquisition of a more metastatic phenotype (21). To further explore the role and significance of cathepsin B overexpression in metastatic melanoma cells, we did functional experiments using specific inhibitors of both cathepsin B (CA-074 Me) and D (pepstatin A). The results showed that treatment of melanoma cells with CA-074 Me significantly decreased the cannibalistic activity of all metastatic melanoma cell lines, whereas pepstatin A was ineffective (Fig. 5*B*), supporting a specific role of cathepsin B in cannibalism of metastatic melanoma cells. These data were consistent with the results of experiments, in which melanoma cells were transfected with cathepsin B siRNA, showing that cathepsin B–silenced cells were clearly unable to ingest lymphocytes compared with untransfected cells (Fig. 5*C* and *D*).

As we have shown previously, ezrin protein, belonging to the ezrin, radixin, and moesin (ERM) actin-binding protein family (22), is involved in the phagocytic activity of human tumor cells (10). We then hypothesized that this protein could also be involved in the tumor cannibalism against live lymphocytes. To this purpose, we

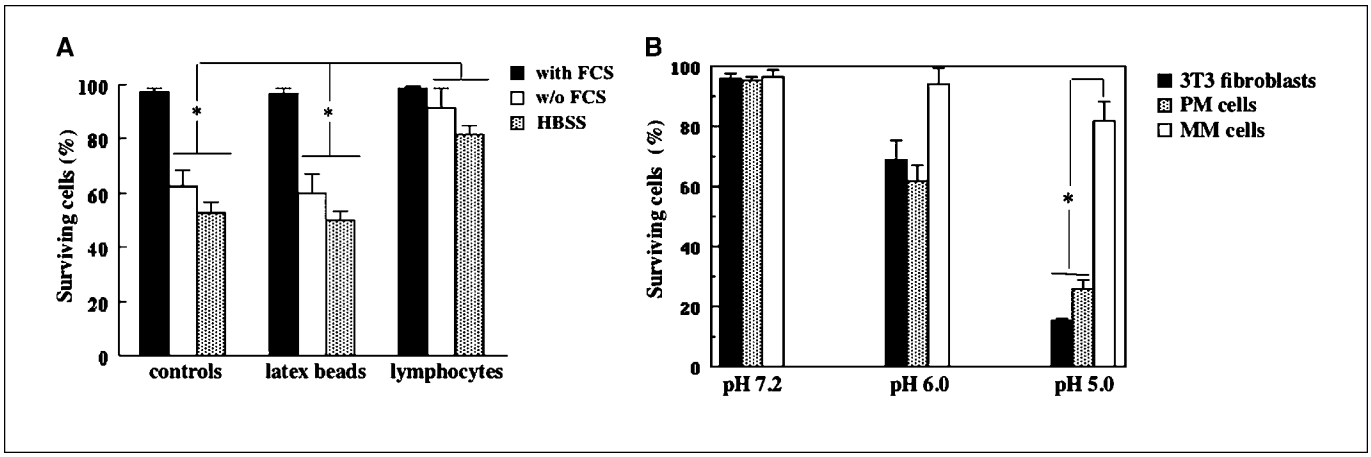

Figure 4. Cannibalism and cell survival. Analysis of cell survival by trypan blue test in different growth conditions. *A,* percentages of metastatic melanoma cells surviving in the presence of FCS (*with FCS*), without FCS (*w/o FCS*), or maintained in HBSS buffer and supplemented with latex beads or with live lymphocytes (melanoma cell to lymphocyte ratio of 1:2.5). Note that the ingestion of lymphocytes conferred to metastatic melanoma cells a significant increase of their survival either in medium without FCS or in HBSS buffer, and the ingestion of latex beads did not increase survival rate. *B,* percentages of surviving 3T3 cells (control fibroblasts; *black columns*), primary melanoma cells (*gray columns*), and metastatic melanoma cells (*white columns*) in different growth medium acidity conditions (pH 7.2, 6.0, and 5.0). *, $P < 0.01$, statistical analyses revealed a significantly higher percentage of surviving metastatic melanoma cells with respect to primary melanoma or 3T3 cells at lowest pH value (5.0).

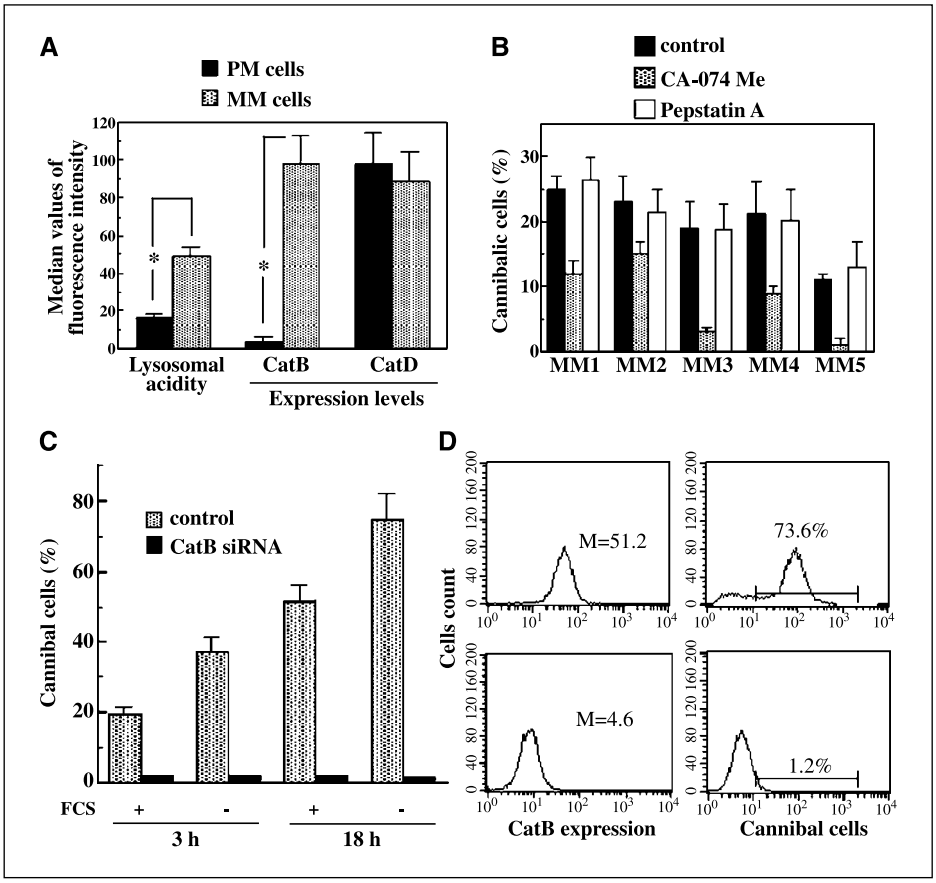

Figure 5. Role of lysosomal acidity and cathepsin B in cannibalistic activity. *A,* semiquantitative cytofluorimetric analysis of lysosomal acidity, cathepsin B (*CatB*), and cathepsin D (*CatD*) expression in primary melanoma cells (*black columns*) and metastatic melanoma cells (*gray columns*). *Columns,* mean of median values of fluorescence intensity histograms obtained in three different experiments; *bars,* SD. *, $P < 0.01$, statistical analyses revealed a significant difference in lysosomal pH and cathepsin B expression between metastatic and primary melanoma cells. By contrast, no difference was detected in cathepsin D expression. *B,* cytofluorimetric evaluation of cannibalistic activity in five different metastatic melanoma cell lines in the presence (*gray columns*) or absence (*black columns*) of the cathepsin B inhibitor CA-074 Me or the cathepsin D inhibitor pepstatin A (*white columns*). Statistical analyses showed that only cathepsin B inhibitor significantly ($P < 0.01$) inhibited cannibalistic activity in all metastatic melanoma cell lines. *C,* time course of cannibalistic activity (melanoma cell to lymphocyte ratio of 1:2.5) in metastatic melanoma (*MM2*) cell line, untransfected (*gray columns*) or transfected with cathepsin B siRNA (*black columns*) and maintained in culture medium with or without FCS for 3 or 18 hours. *Columns,* mean of three different experiments; *bars,* SD. *D,* percentages of cannibalistic cells (*right*) in control (*top*) and cathepsin B siRNA-transfected melanoma cells (*bottom*) compared with cathepsin B expression (*left*) in the same cells (numbers are median values of fluorescence intensity as analyzed by flow cytometry). Representative of three independent experiments.

transfected two metastatic melanoma cell lines with an ezrin deletion mutant and compared the cannibalistic activity of the transfected cells with that of the untransfected cell line (WT). The percentage of melanoma cells engulfing live lymphocytes was significantly ($P < 0.01$) reduced in the ezrin mutant-transfected cells with respect to WT cells (Fig. 6*A*). These results were confirmed by a different technique, in which melanoma cells, either WT or ezrin mutant, were labeled with the phagocytic marker PKH26 and then analyzed for their ability to internalize CD8-stained T lymphocytes at different melanoma cell to lymphocyte ratio. In fact, as depicted in Fig. 6*B* and *C*, cannibalistic activity toward T cells was significantly higher in WT with respect to ezrin mutant melanoma cells (Fig. 6*B*). Further analyses showed that the cannibalistic activity of melanoma cells was higher at the lowest lymphocyte concentration (Fig. 6*B*) and that the ezrin mutant melanoma cells were more susceptible to the lytic activity of the autologous $CD8^+$ T cells (Fig. 6*C*). The statistical analysis of these experiments showed that the cannibalistic activity of melanoma cells was inversely correlated to their susceptibility to the lytic activity of $CD8^+$ T cells (Fig. 6*D* and *E*).

To further define the mechanisms of melanoma cannibalism, we did electron microscopy examination, indicating that a recruitment of well-defined caveolar structures occurred in the sites of lymphocyte-to-tumor cell interaction (Fig. 7*A* and *B*). Several caveolar-like vesicles, clearly distinguishable from the classic clathrin-coated vesicles and fusing between them, were also detectable in tumor cell cytoplasm (Fig. 7*C* and *D*). These observations suggested that a caveolae-dependent pathway could participate to the formation of the phagocytic vesicles. In fact, treatment of metastatic cells with filipin (an antibiotic that disrupt rafts and caveolae; ref. 23) showed a clear dose-response inhibition of the cannibalistic activity of melanoma cells against the autologous T lymphocytes (Fig. 7*E*). Based on previous reports on the role of actin and ERM proteins on the formation and maintenance of lipid rafts structure (24), we tested the possible association between ezrin and caveolin-1 in metastatic melanoma cells. Western blot analysis showed that caveolin-1 is enriched in the endolysosomal compartment of cannibal cells and that caveolin-1 is detectable in ezrin immunoprecipitates obtained from the endolysosomal compartment (Fig. 7*F*). Conversely, ezrin and actin were detectable in caveolin-1 immunoprecipitates of the same fraction (Fig. 7*F*). These results suggested that caveolin-1-rich structures represent a major component of the endolysosomal compartment of cannibal tumor cells and that, in this compartment, ezrin, actin, and caveolin-1 proteins are associated. Recent data have suggested that ezrin has a key role in the metastatic behavior of tumors (25, 26). Our data suggest that ezrin provides metastatic tumor cells with an altered connection between actin and caveolin-1-enriched vacuoles, which is the driving structure of the cannibalistic process.

Discussion

Cell cannibalism is defined as the ability of a cell to phagocyte another cell. In humans, this phenomenon has been recognized as an exclusive property of malignant tumor cells (1–9), whereas normal human cells seem to be equipped to exert self-cannibalism that is a sort of "self-eating" in condition of nutrient

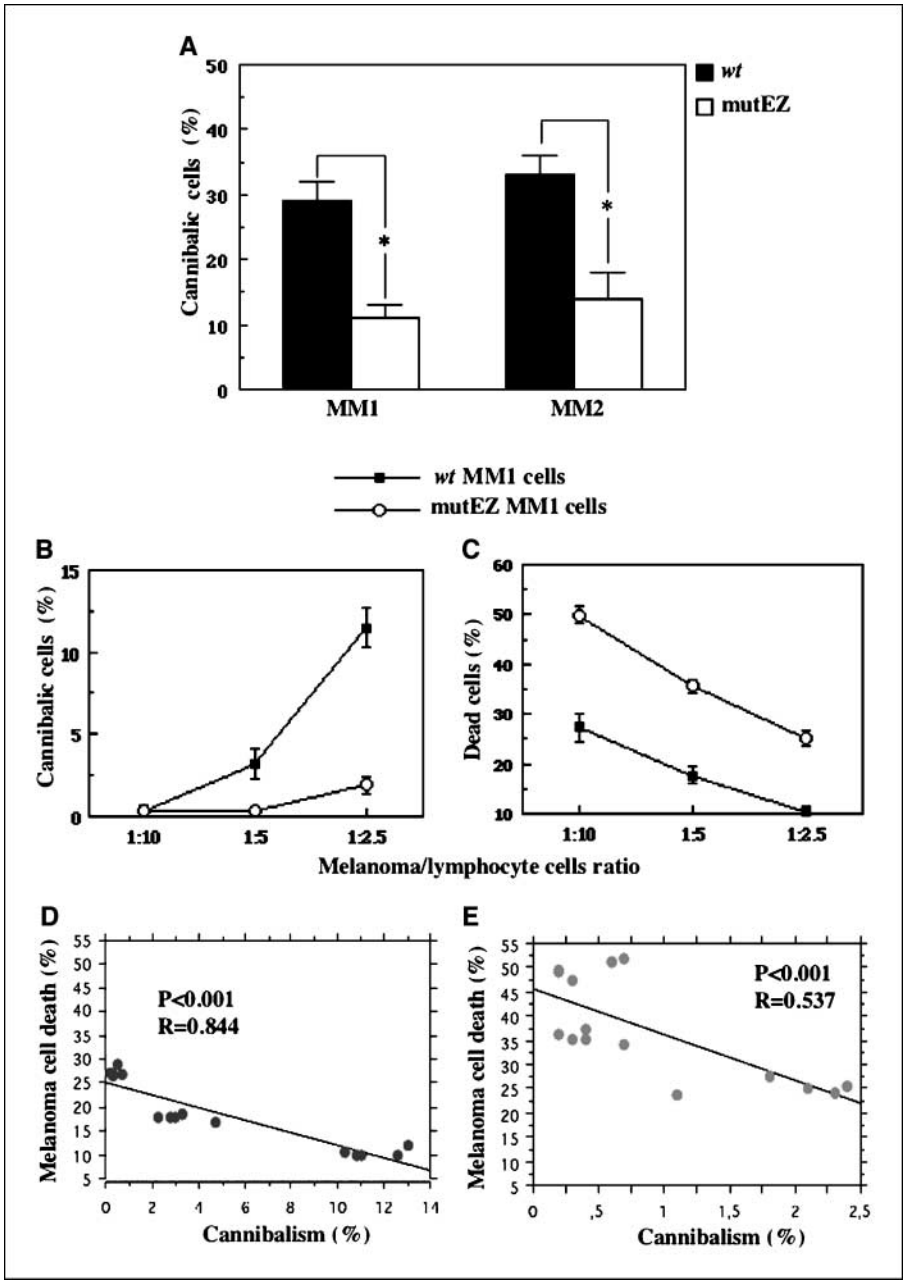

Figure 6. Role of ezrin in cannibalistic activity. *A,* cytofluorimetric evaluation of cannibalistic activity of two representative metastatic melanoma cell lines (*MM1* and *MM2*; *wt, black columns*) compared with the same cell lines transfected with mutated ezrin (*mutEZ, white columns*). *Columns,* mean of four independent experiments; *bars,* SD. *, $P < 0.01$, statistical analyses revealed that the percentage of cannibal cells was significantly higher in WT cells compared with the ezrin mutant-transfected counterparts for both cell lines considered. *B,* cytofluorimetric quantification, after labeling of metastatic melanoma cells with the phagocytic marker PKH26, of the capability of WT cells versus ezrin mutant metastatic melanoma cells to internalize autologous $CD8^+$ T cells at different melanoma cell to lymphocyte ratios. Statistical analyses of data indicated a significantly higher ($P < 0.01$) cannibalistic activity in WT cells with respect to ezrin mutant metastatic melanoma cells. *Columns,* mean of four independent experiments; *bars,* SD. Note that the higher the rate of lymphocytes, the lower the rate of cannibalistic activity of metastatic melanoma cells. *C,* standard 4-hour ^{51}Cr release assay of the cytotoxicity of autologous $CD8^+$ T cells against metastatic melanoma cells (both WT and ezrin mutant) at different melanoma cell to lymphocyte ratios. Statistical analyses indicate a significant ($P < 0.01$) difference, in the percentage of cell death, between WT and ezrin mutant metastatic melanoma cells: the higher the number of lymphocytes, the higher the rate of dying melanoma cells. Regression analysis of these two phenomena points to an inverse correlation either in WT (*D*; $P < 0.01$; $r = 0.844$) or in ezrin mutant (*E*; $P < 0.001$; $r = 0.537$) metastatic melanoma cells. This clearly indicates a close relationship between the two events: the ability of metastatic melanoma cells to counteract the cytolytic activity of $CD8^+$ T cells (and to cannibalize them) is strictly related to the melanoma cell to lymphocyte ratio.

deprivation (27). This study adds to our understanding on human tumor cannibalism in several ways. First, we have shown that cannibal cells can be detected in 100% metastatic melanoma lesions and that cannibal cells contain not only sister or sibling cells but also T lymphocytes, conceivably those that should potentially kill them. T lymphocytes contained in the tumor cells showed *in vivo* degradative phenomena, suggesting their progressive digestion after the early engulfment. We have shown previously that metastatic melanoma cells are able to phagocytose apoptotic cells of various origins (10), but we ignored whether this phagocytic activity could involve also live cells, particularly T lymphocytes. Using a model of cocultivation of metastatic melanoma cells with the autologous T lymphocytes (11), we have shown that T cells, after an early contact, were endocytosed by tumor cells. Our data also indicate that the cannibalistic process significantly differs from the typical phagocytosis exerted by professional phagocytes and is characterized by the presence of surface ruffles surrounding the external body undergoing engulfment (16). Cannibalism of live lymphocytes occurs via a peculiar mechanism that suggests a sort of "sinking" of the live lymphocyte into the tumor cell. This lead, in the early steps, to the formation of large vacuoles, where the lymphocyte remains alive. At later time points, engulfed cells progressively undergo necrosis and degeneration. We quantified this phenomenon in comparing metastatic melanoma cells with primary melanoma cells and primary macrophages, showing that only metastatic melanoma cells were able to cannibalize live T lymphocytes. We have also shown that cannibalism is markedly increased under conditions of serum starvation and that the ingestion of live lymphocytes is related to tumor cell survival, thus suggesting that cannibalism may represent a survival option of tumor cells under low nutrient supply. Metastatic melanoma

cannibal cells remain alive in acidic medium, supporting the concept that the microenvironment has a key role in the selection of malignant cell clones able to survive in the unfavorable conditions established by the low blood supply (i.e., hypoxia and acidity).

To understand more on the mechanisms underlying tumor cannibalism, we have explored two additional aspects related to this phenomenon: (*a*) the digestive machinery of cannibal cells and (*b*) the role of proteins known to modulate the interaction between tumor cells and their microenvironment. The results have shown that cannibal cells are characterized by an increased acidity of lysosomal-like vesicles and an overexpression of cathepsin B, a proteolytic enzyme reported to be involved in tumor invasion and metastasis (21). Suppression of cathepsin B activity through either specific inhibitors or siRNAs leads to a marked decrease in the cannibalistic activity of metastatic melanoma cells. One of the major mechanisms involved in the acidification of intracellular organelles is the expression of vacuolar ATPases (19) markedly expressed and continuously activated in metastatic melanoma cells (18). Moreover, cathepsin B is among the proteolytic enzymes that are potently activated in acidic conditions (20), suggesting that metastatic melanoma cells are continuously predisposed to digest even complex materials through a vacuolar ATPases–mediated acidification of intracellular organelles, which in turn may maintain cathepsin B in an activated condition (20).

In investigating the leading mechanism of the cannibalistic process, we started from three important observations: (*a*) electron microscopy showed a recruitment and fusion of caveolar-like structures in the points where the lymphocytes and melanoma cells established an early contact, (*b*) our previous finding that ezrin was involved in the phagocytic activity of metastatic melanoma cells against apoptotic cells (10), and (*c*) ezrin and caveolin-1 are generally involved in the metastatic behavior of tumors (25, 26, 28). Thus, we did a series of experiments aimed at defining the role of caveolar structures in this phenomenon and the possible connection of caveolae to ezrin in forming a dynamic structure. Caveolae were identified morphologically a long time

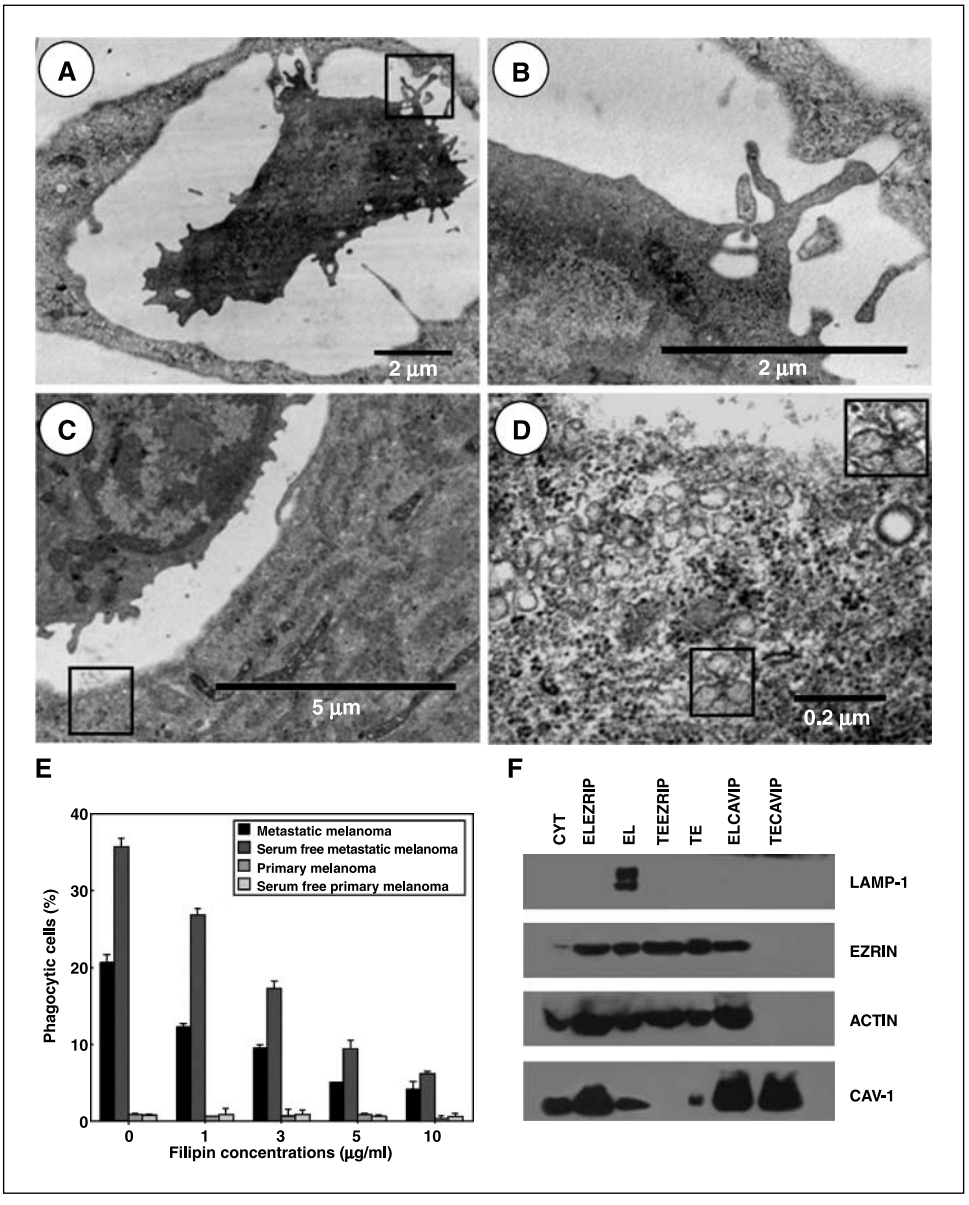

Figure 7. Role of caveolae in cannibalistic activity. *A* to *D,* TEM analysis of an internalized lymphocyte by a metastatic melanoma cell. *A,* cannibalized lymphocyte undergoing degeneration. *B,* higher magnification of the boxed area in (*A*). *C,* recruitment of caveolae-like vesicles in the cannibalistic melanoma cell cytoplasm. *D,* higher magnification of the boxed area in (*C*). A large caveolae-like structure. Coalescence of caveolae-like vesicles can also occur [*inset,* higher magnification of the boxed area in (*D*)]. *E,* filipin treatment inhibits in a dose-dependent manner the cannibalistic activity of metastatic melanoma cells in either the presence (*black columns*) or the absence (*dark gray columns*) of FCS in the culture medium. *F,* Western blot analysis of the expression of ezrin and caveolin-1 protein (*CAV-1*) in total cellular extract (*TE*), endolysosomal compartment (*EL*), and cytosol (*CYT*) of representative metastatic melanoma cells. Caveolin-1 is present in endolysosomal ezrin immunoprecipitate (*ELEZRIP*) but not in total cellular extract ezrin immunoprecipitate (*TEEZRIP*), and mutually, ezrin and actin are detectable in endolysosomal caveolin-1 immunoprecipitate (*ELCAVIP*), such as actin, but not in total cellular extract caveolin-1 immunoprecipitate (*TECAVIP*). Purity of endolysosomal compartment fraction is confirmed by the presence of LAMP-1.

before the identification of caveolin proteins as defining protein components of these structures (29). Moreover, the existence of alternative pathways of endocytosis occurring through caveolae (30) was described to be involved in the pathogens entry into the cells (31, 32). The results of our investigation showed clearly that the endolysosomal compartment of the cannibal cells was very rich in caveolin-1. Moreover, in the same subcellular fraction, caveolin-1, actin, and ezrin were associated, suggesting a connection between caveolin-1 and the actin cytoskeleton through ezrin. This conclusion was further supported by the fact that treatment of melanoma cells with actin depolymerizing agents, such as cytochalasin B or latrunculin, virtually abolished the cannibalistic activity of melanoma cells (data not shown).

Altogether then, it seems that the driving force of the "cannibalistic vacuole" is represented by a simple and highly efficient mechanism through which any live or dead material that touches the tumor cell external membrane is immediately endocytosed and digested through a sort of "quicksand" mechanism. A membrane-to-cytosol framework, including a dynamic link between caveolin-1, ezrin, and actin, seems to have a key role in the formation of the "cannibalistic vacuole." This connection allows the formation of caveolae-enriched endosomes, which we can call caveosomes (30), and that seem to represent the driving structure of cannibalism. These caveosomes contain a simple but highly efficient digestive machinery as represented by an acidic milieu and a potent proteolytic enzyme, such as cathepsin B. In fact, inhibition of the activity of the various actors of tumor cannibalism, including ezrin, caveolae, and cathepsin B, has led to substantial inhibition of cannibalistic activity. Moreover, transfection of tumor cells with an ezrin mutant impaired the cannibalistic activity while allowing tumor-specific CTLs to fully exert their cytotoxic effect. This suggests that actions aimed at inhibiting tumor cannibalism may represent a new and useful approach to improve the efficacy of antitumor immunotherapies. It seems, however, conceivable that inhibition of tumor acidity through specific agents (18) may represent a further therapeutic approach against cannibalistic activity. Moreover, our study shows that the cannibalistic activity of metastatic tumor cells increased under serum-free culture and that the melanoma cells from metastatic lesions survived in acidic conditions. This, in turn, suggests that metastatic tumor cells may be selected within the tumor mass in a microevolutionary way due to the environmental conditions. The selected tumor cells behave as unicellular microorganisms, such as amoebae and *Bacillus subtilis* (33, 34), which use their eating to feed, whereas professional phagocytes in higher animals use their eating to scavenge the body from necrotic material and debris and to initiate the immune response (35).

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