

Research Article

The Metastatic Potential of Human Pancreatic Cell Lines in the Liver of Nude Mice Correlates Well With Cathepsin B Activity

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Abstract

Background. Cathepsin B, a lysosomal cysteine protease, has a major role in the mechanisms of tumor metastasis. The aim of the present work was to examine the correlation between cathepsin B activity and the metastatic potential of human pancreatic cancer.

Methods. The primary cell line COLO 357 and the derivative tumor cell lines FG, L3.1, L3.2, L3.3, L3.4, and L3.5, which are characterized by progressively increasing metastatic potential, were injected intrasplenically in the athymic mice. Cathepsin B activity, metastasis, and ultrastructural characteristics were assessed.

Results. An increased number of liver tumor nodules was observed with each subsequent intrasplenic inoculation ($p = 0.001$), associated with lymph node, splenic, and pancreatic involvement. Cathepsin B activity progressively increased ($p = 0.001$) and was strongly positively correlated with the metastatic potential. However, no correlation was found between the metastatic potential and ultrastructural characteristics.

Conclusions. These findings further support the central role of cathepsin B in metastasis in a combined in vitro/in vivo model.

Key Words: Pancreatic carcinoma; metastatic potential; cathepsin B; in vitro/in vivo model; ultrastructural characteristics.

Introduction

Multiple lines of evidence suggest that cathepsin B, a cysteine protease, plays an important role in the earlier events of tumor development, including tumor

cell proliferation, neoangiogenesis, and metastasis (1,2). Cathepsin B is present in lysosomes and the surface of cancer cells (3,4). Its posttranslational processing results in a product of 30 kDa that represents the active form (5). The latter translocates to the plasma membrane, where it presumably participates in the degradation of several extracellular matrix components, including laminin, fibronectin, and collagen IV (6–8). It is hypothesized that these effects in combination with other proteases activate a proteolytic cascade that promotes tumor invasion (6).

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Cathepsin B activity has been studied in breast (9), colorectal (10), gastric (11), lung (12), and prostate carcinomas (13), melanomas (14), osteoclastomas (15), and gliomas (16).

In pancreatic cancer, cathepsin B expression appears to be a prognostic factor (17). However, quantitative and qualitative *in vivo* studies on the significance of cathepsin B in pancreatic carcinomas are difficult to perform because of the lack of an appropriate experimental model. Heterotransplantation of neoplasms into the athymic mouse has provided an animal model that facilitates investigation of human cancers (18–20). The isolation of COLO 357—a metastatic pancreatic cell line with unique properties including tumor-associated proteins, protease enzymes, a distinctive karyology, and an allozyme profile (21)—provides a tumor cell line to elucidate the pathogenesis of metastasis in the athymic mouse. The aim of our study was to examine if metastatic potential, ultrastructural characteristics, and cathepsin B activity correlated in a graded *in vivo/in vitro* model of pancreatic adenocarcinoma. We opted for activity over expression and/or localization based on reports showing that high cathepsin B activity correlates well with the size of rat colon tumors (22) and that experimental liver metastases of colon cancer cells can be reduced with a selective inhibitor of extracellular cathepsin B activity (23).

The pancreatic carcinoma cell line COLO 357 and its sublines were used because of their progressively increased metastatic potential (24). Each cell line, following stabilization in culture, was injected intrasplenically into athymic NCI nude mice, and the number and size of metastases were quantitated and correlated to cathepsin B activity.

Materials and Methods

Cell Cultures

COLO 357 was established as a cell line by Morgan et al. (21) from a lymph node metastasis of a human pancreatic adenocarcinoma, and its variant cell lines (FG, L3.1, L3.2, L3.3, L3.4, and L3.5) have been established by this laboratory (24). The cell lines were maintained in GEM 1717 medium. The medium was prepared using RPMI 1640 (Gibco, Grand Island, NY), which was buffered to pH 7.3 with HEPES (10 mM final concentration), tricine (10 mM), and

sodium bicarbonate (33 mM) (all buffers were obtained from Gibco). The buffered RPMI 1640 was supplemented with heat-inactivated fetal bovine serum (FBS) (10% final concentration, Gibco), protamine, zinc, iletin, cynocobalamin (Lilly, Indianapolis, IN), oxaloacetic acid, and pyruvate (Sigma, St. Louis, MO). Antibiotics added to the medium included penicillin (100 U/mL, Gibco), streptomycin (100 µg/mL, Gibco), fungizone (2.5 µg/mL amphotericine, Gibco), tyrosine (60 µg/mL, Gibco), and garamycin (20 µg/mL, Elkins-Sinn, Richmond, VA). The monolayer cultures grew in a humidified 5% CO₂ atmosphere at 37°C in 25-cm tissue culture flasks (Nunclon, Thomas Scientific, Philadelphia, PA). Cells were fed at least once a week with GEM 1717. When the cell cultures reached confluency (about 1/20 of the culture was passaged prior to trypsinization), they were washed with 0.9% saline (containing 100 U/mL penicillin and 100 µg/mL streptomycin), and trypsinized with 0.25% trypsin in 0.1% EDTA (pH 7.3) (both from Gibco) for 15 min at 37°C. Trypsin was inactivated by addition of GEM 1717 containing 10% FBS. Cells were immediately centrifuged gently at 100g for 5 min and resuspended in the fresh medium. The tumor cells were grown in 100-mm-diameter petri dishes (Nuclon). Cultures were harvested as above and resuspended after centrifugation into Hanks balanced salt solution (Sigma). Cells were counted using a hemocytometer, and viability was assessed with trypan blue dye exclusion.

In Vivo Experiments in Athymic NCI Nude Mice

Six-week-old athymic NCI nude mice (NCR-nu/nu) were used as the experimental animals. They were maintained in a positive-pressure isolation room at the animal care facility of the Providence Veterans Administration Medical Center in Rhode Island. The food, water, and bedding for the mice were sterilized and changed at least once weekly. Surgical gowns and gloves were used when working in the room to prevent pathogenic contamination. A total of 138 animals were used. Sixty-eight animals (7–12 animals per group) were used to assess the metastatic potential of the various cell lines. We used the model of intrasplenic tumor cell injections as described previously (24). Briefly, following the establishment of satisfactory anesthesia, the

abdomen was entered through a midline incision approx 1 cm in length. The spleen was exteriorized by applying gentle traction, and 2×10^6 cells suspended in 0.02 mL of Hank's balanced salt solution were injected just under the capsule of the lower pole of the spleen using a tuberculine syringe with a 30-ga needle (Ethicon, Somerville, NJ). Hemostasis was obtained, the spleen was returned to the peritoneal cavity, and the incision was closed in two layers using a continuous 4-0 Vicryl suture (Ethicon) for the muscles and clips (American Hospital Supply) for the skin. The animals were allowed to recover and the skin clips were removed 10 d after the operation. The animals were sacrificed by craniocervical dislocation 30 d postoperation. Autopsies were performed and the findings were recorded. Livers, spleens, lymph nodes, and other organs suspected of harboring metastases were harvested and fixed in phosphate-buffered 10% formalin (Fisher, Pittsburg, PA). Seventy animals (10 animals per group) were injected subcutaneously with 1×10^7 cells of one cell line. The animals were sacrificed 30 d later by craniocervical dislocation. Autopsies were performed; the subcutaneous tumor numbers, weight, and diameters were recorded; and the tumors were harvested for the determination of cathepsin B activity.

Determination of Cathepsin B Activity

Tumors harvested from subcutaneous injections 2 g/cell line, were homogenized in 10 mL of 0.25 M sucrose. The total protein concentration of the homogenates was determined as described by Lowry et al. (25). Lysosomal cathepsin B activity in the tumor homogenates was measured by monitoring the hydrolysis of the preferential substrate, carbobenoxyl-alanyl-arginyl-arginine 4-methoxy-B-naphthylamide (Enzyme Systems Products, Inc., Livermore, CA) fluorometrically (26,27). Determinations in quintuplicate were conducted with a 65- μ L aliquot of the tumor homogenate incubated at 37°C for 2 h with the substrate (35 μ L of a 29.3-mmol/L solution) in a 40-mmol/L mixed sodium citrate-sodium phosphate buffer (pH 6.0) that contained 2 mmol/L dithiothreitol and 8 mmol/L EDTA. The reaction was carried out with a total volume of 2 mL and was stopped by the addition of 1.0 mL of 1.0 N hydrochloric acid. Fluorescence was read using a Perkin-Elmer MPF-44B spectrophotofluorometer with excitation at 292 nm and

emission at 425 nm against a standard curve with different concentration of 4-methoxy-B-naphthylamine (27,28).

Electron Microscopy: Assessment of Ultrastructural Characteristics

For electron microscopy, trypsinized cell suspensions from the various cell lines were fixed in a modified Karnovsky's fixative containing 1.8% paraformaldehyde and 2.5% glutaraldehyde and postfixated for 1 h with 2% aqueous OsO₄. Following osmication, the suspensions were centrifuged to pellet the cells. The supernatant was removed, and 2.5% agarose at 45°C was added. Resuspended cells were centrifuged, and the tubes were chilled at 4°C for 15 min to harden the agarose. Agarose blocks containing the cells were trimmed with razor blades, dehydrated with graded ethanol, cleared in propylene oxide, and embedded in an Epon-Araldite mixture. Semithin plastic sections, cut with glass knives, were stained with 1% toluidine blue, and representative areas were selected for ultrastructural study. Ultrathin sections, cut with diamond knives, were stained with uranyl acetate and lead citrate. Sections were viewed and photographed in a Philips 300 transmission electron microscope.

Statistical Analysis

The results were expressed as median (range) and as mean \pm SD. Statistical analysis was performed by a one-way analysis of variance or an unpaired *t*-test to determine significant differences among groups. Simple linear regression and correlation analysis was used to assess the relation between the number of liver nodules and cathepsin B activity in respective groups. *p* values <0.05 have been considered statistically significant.

Results

Quantification of the Metastatic Capacity of Pancreatic Adenocarcinoma Cell Lines Following Intrasplenic Injection in Nude Mice

Increased hepatic metastases were observed with each subsequent selection and intrasplenic inoculation of the cell lines (Fig. 1). Analytically, the primary tumor cell line, COLO 357, and the FG cells

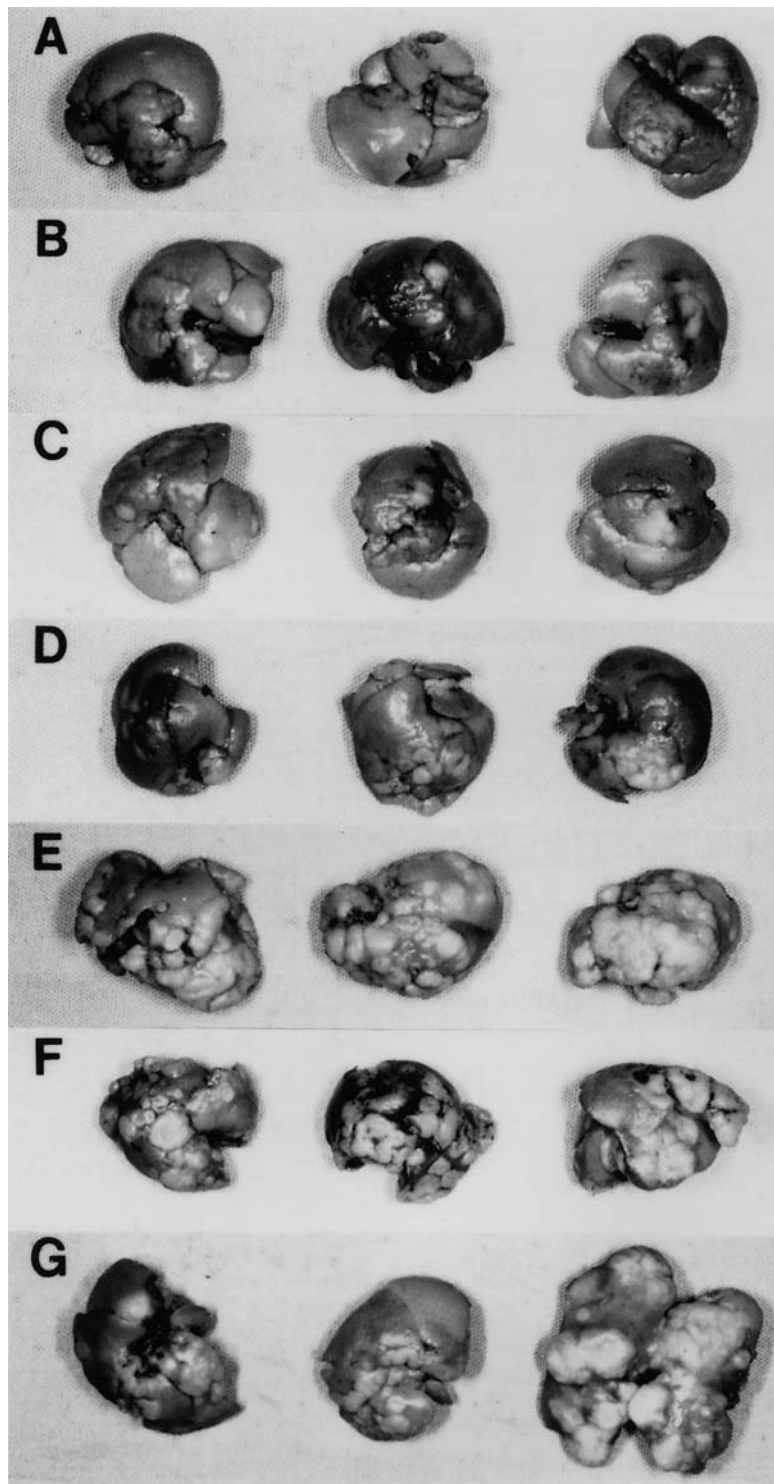


Fig. 1. Tumors growing in the livers of athymic mice whose spleens had been injected with various human pancreatic cancer cell lines. (A) COLO 357, (B) FG, (C) L3.1, (D) L3.2, (E) L3.3, (F) L3.4, (G) L3.5.

Table 1
Selection of Tumor Cell Lines With Increased Metastatic Potential From a Human Pancreatic Carcinoma (COLO 357)

Tumor cell lines	In vitro doubling time (h)	No. of mice ^a with hepatic metastases/total mice	Median hepatic nodules/mouse (range)	No. of mice with splenic tumors/total mice	Regional mesenteric lymph node metastasis	Other visceral metastasis
COLO 357	22–2	5/7	3 (0–4)	0/7	1/7	0/7
FG	32–5	7/10	4 (0–6)	0/10	1/10	0/10
L3.1	33	6/8	11 (3–16)	3/8	0/8	0/8
L3.2	–	8/10	12 (5–19)	6/10	5/10	1/10
L3.3	37	10/12	22 (8–52)	8/12	7/12	1/12
L3.4	17	8/10	28 (13–53)	7/10	7/10	5/10
L3.5	21	11/11	34 (14–45)	6/11	8/11	4/11

^a 2×10^6 viable cells were injected into the spleens of athymic mice, and metastatic potential was determined by production of metastases to the liver and other organs.

produced the lowest median number of liver nodules, 3 and 4 per liver, respectively. The L3.1 (median: 11 nodules) L3.2 (median: 12) L3.3 (median: 22) L3.4 (median: 28), and L3.5 cells (median: 34) showed a progressive increase in the number of liver nodules ($p = 0.001$) (Table 1). Splenic metastases were not observed in the COLO 357 or FG groups but were observed in three, six, eight, seven, and six animals in the L3.1, L3.2, L3.3, L3.4, and L3.5 groups, respectively. The COLO 357 and FG cells each produced visible mesenteric lymph node metastases in only one animal, whereas the L3.2, L3.3, L3.4, and L3.5 cells produced such metastases in five, seven, seven, and eight mice, respectively. Pancreatic metastasis in one mouse was observed with each of the L3.2 and L3.3 cells. L3.4 and L3.5 cells produced pancreatic metastases five and four mice, respectively.

Growth of Pancreatic Adenocarcinoma Cell Lines After Subcutaneous Implantation

The growth of human pancreatic cell carcinomas following subcutaneous injection of each of the tumor cell lines in athymic mice resulted in growth of all implantations (Table 2). The median tumor weight in the COLO 357 group was 0.4 g. The median tumor weight produced was 1.0, 0.8, and 1.2 g by the IG, L3.1, and L3.2 cells, respectively. The median

weight of subcutaneous tumors produced by the L3.3, L3.4, and L3.5 cells was approx 2.2 g for each cell line. The median tumor diameter correlated with the weight of tumors produced by the various cell lines.

Cathepsin B Activity of Tumor Cell Lines

As shown in Fig. 2, the lowest cathepsin B activity levels are present in the COLO 357 cells, 695 pmoles of 4-methoxy B-naphthylamide/mg protein/minute. The tumors produced by the selected cell lines have progressively increased cathepsin B activities ($p = 0.001$). Namely, the FG, L3.1 and L3.2 cells produce 1415, 2133, and 2265 pmoles of 4-methoxy B-naphthylamide/mg protein/minute, respectively. Even higher cathepsin B activities are observed in tumors produced by the L3.3, L3.4, and L3.5 cells at 2953, 2573, and 2900 pmoles of 4-methoxy B-naphthylamide/mg protein/minute. Cathepsin B activity was positively correlated with the number of liver tumor nodules ($r = 0.866$, $p = 0.0117$).

Electron Microscopy Data

The tumor cells from all sublines studied by electron microscopy had similar morphological features. The large nucleoli appeared compact, and the cytoplasm contained polyribosomes, tonofilaments, small filiform mitochondria, and occasional pools

Table 2
Growth of Human Pancreatic Cell Carcinoma After Subcutaneous Implantation in Athymic Mice.^a

Tumor cell lines	Incidence at sc site ^b	Median tumor weight, g (range)	Median tumor diameter, mm (range)
COLO 357	7/10	0.40 (0–0.7)	5.5 (0–7)
FG	10/10	1.02 (0.7–1.5)	17.0 (10–27)
L3.1	10/10	0.80 (0.4–1.4)	17.5 (12–26)
L3.2	10/10	1.20 (0.7–2.3)	20.5 (12–25)
L3.3	10/10	2.20 (1.1–3.2)	30.5 (23–32)
L3.4	10/10	2.10 (0.8–2.8)	30.5 (12–36)
L3.5	10/10	2.30 (1.2–3.5)	33.0 (14–38)

^aMice were injected with 1×10^7 viable cells.

^bsc, subcutaneous.

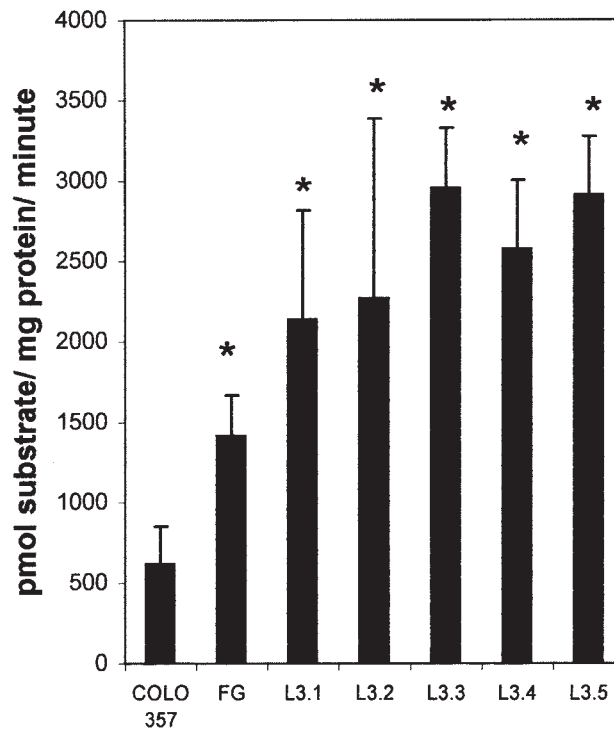


Fig. 2. Cathepsin B activity was measured in pmoles of 4-methoxy B-naphthylamide produced/milligram protein/minute. Statistical significance was determined by an unpaired student's *t*-test, where * denotes $p < 0.05$ as compared to the COLO 357 cell line.

of glycogen (Fig. 3). The rough endoplasmic reticulum and golgi complexes were inconspicuous. Intracytoplasmic lumina lined by microvilli were focally present in every group, and nearly all cells displayed abundant surface microvilli lacking core rootlets without a discernable glycocalyx. Despite

the overall homogeneity of these cells, some features unique to certain groups were noteworthy. Lysosomal residual bodies were especially abundant in the L3.4 group, often filling a considerable portion of the cytoplasm (Fig. 4), and were seen, though to a far lesser degree, in the L3.3 and L3.5

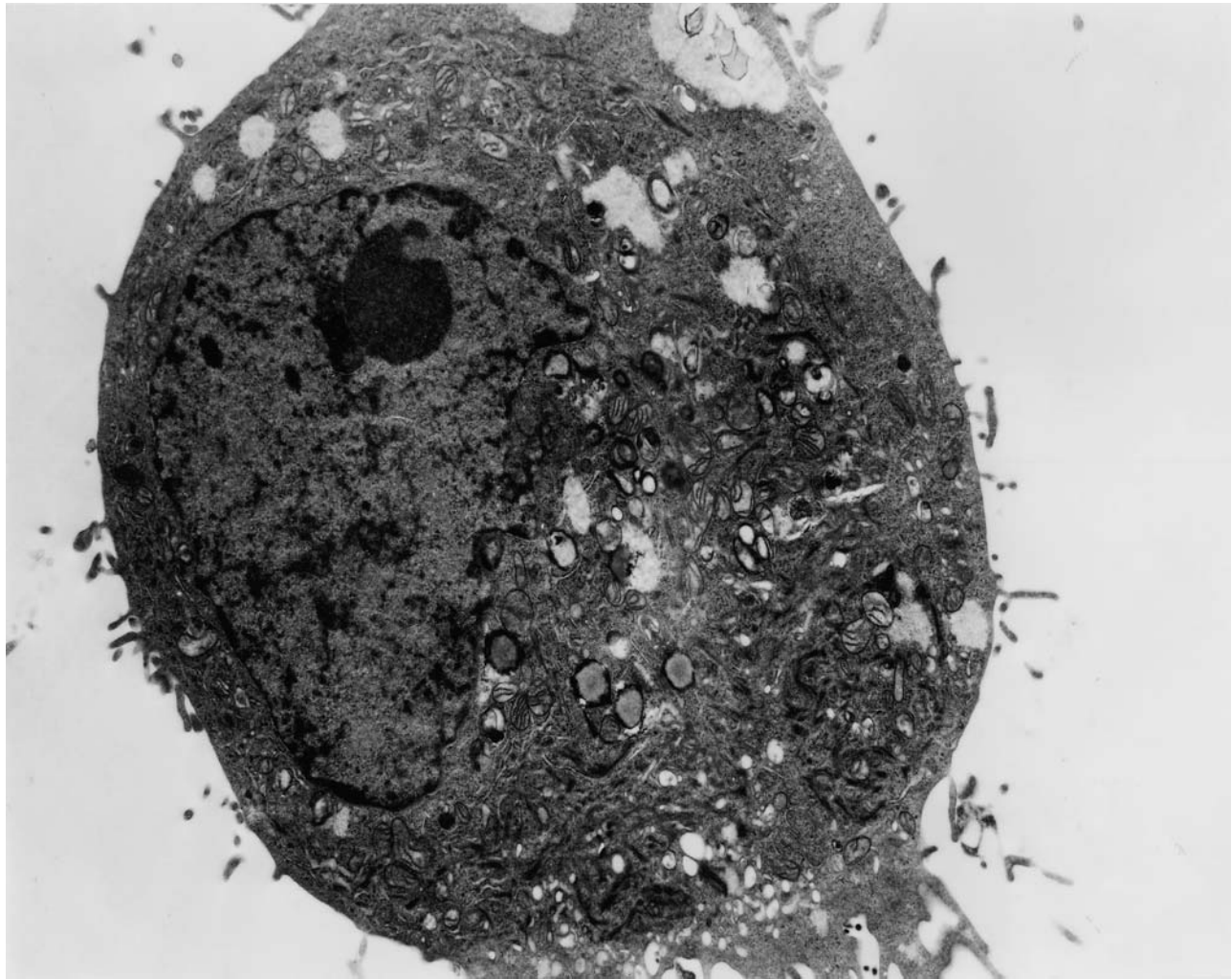


Fig. 3. COLO 357 cells showing tonofilaments, pale lipid droplets, small pools of glycogen, and nuclei with large nucleoli. ($\times 13050$)

cell lines. Focal multivesicular bodies were observed in the L3.3 and L3.5 groups. Polyribosomes were prominent in the L3.1, L3.2, and L3.5 groups (Fig. 5), which also displayed fewer organelles than the other groups.

Discussion

Our current work in an *in vitro/in vivo* model of human pancreatic tumors adds to the evidence that cathepsin B activity directly correlates to the metastatic potential. In this study, we exploited a unique quality of the COLO 357 cell line and its FG, L3.1, L3.2, L3.3, L3.4, and L3.5 subline derivatives, having

graded metastatic potentials that were quantitated in athymic NCI mice inoculated by 2×10^6 cells under the capsule of the lower pole of the spleen. Our data indicate that each cell line exhibits a distinct metastatic potential, which correlates positively with its characteristic cathepsin B activity. More specifically, the activity of cathepsin B was progressively elevated in sublines FG, L3.1, and L3.2 on an average 2-, 3.1-, and 3.3-fold, respectively, as compared to the original COLO 357 cell line. The hypothesis that cathepsin B activity is directly related to metastatic potential is in agreement with multiple published reports documenting a direct relationship between the aggressivity of a tumor, its metastatic potential,

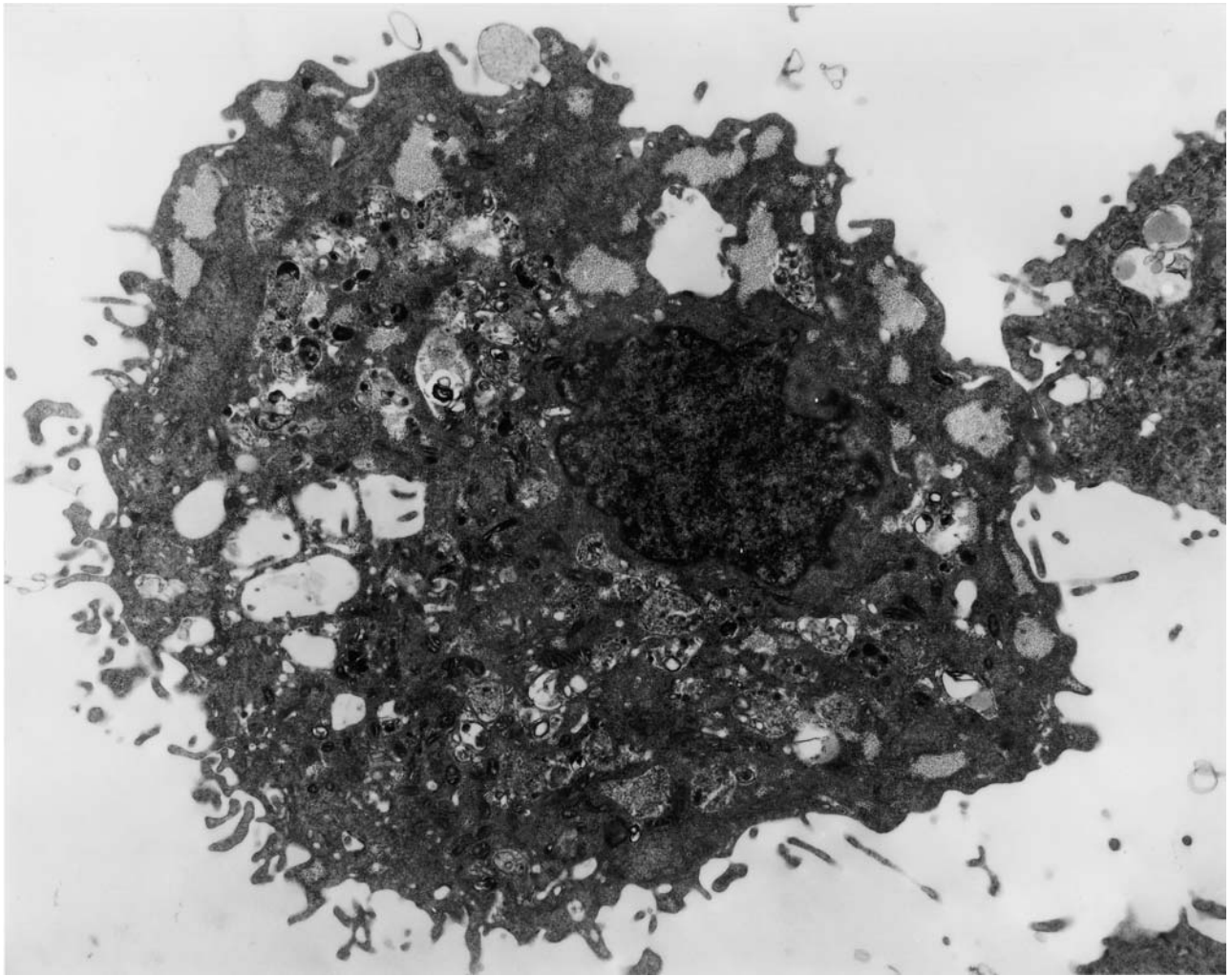


Fig. 4. L3.3 cells showing numerous multivesicular bodies and small pools of glycogen. Structures suggesting intracytoplasmic lumina are also present. ($\times 16240$)

and cathepsin B expression, activity, and subcellular localization (22,23,29–32). Thus, Sloane et al. (14) found that cathepsin B activity is significantly elevated in variants of the B16 melanoma that have high metastatic potential and suggested that cathepsin B may be a focus for therapeutic intervention in the metastatic cascade. Poole et al. (33) reported that cathepsin B production is significantly higher in malignant than in nonmalignant breast tissues. Rempel et al. (34) demonstrated a positive correlation between levels of cathepsin B in human gliomas and invasiveness as assessed by clinical criteria and magnetic resonance imaging. High cathepsin B levels have been associated with significantly shorter overall survival of patients with colon cancer, suggest-

ing that cathepsin B plays an important role in the progression of colorectal tumors (10). Similarly, high cathepsin B levels have been reported to be a significant indicator for decreased disease-free periods in patients with head and neck cancers (35). In ductal pancreatic carcinoma, cathepsin B serum concentrations are significantly elevated (36), and cathepsin B expression in tissue samples from patients with adenocarcinoma of the pancreas predicts survival time following surgery (17). Our data corroborate these bibliographic findings and offer an *in vivo* model for the study of pancreatic tumor invasiveness and cathepsin B production/activity. Our findings are significant because we compared metastatic potential and tumor cathepsin B activity in the same tumor (the

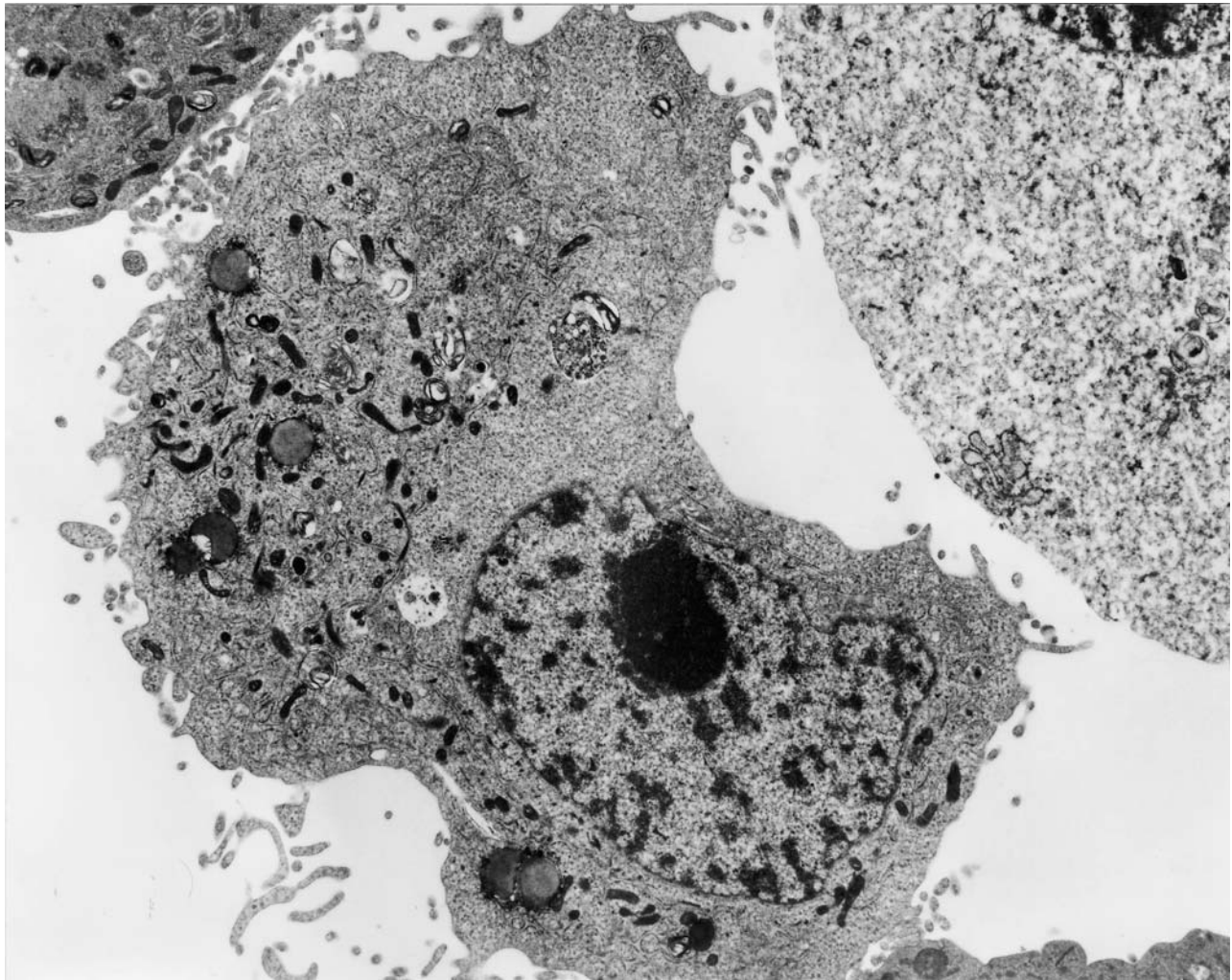


Fig. 5. L3.5 cells with abundant polyribosomes and relatively few organelles (small, dense mitochondria, lipid droplets, and multivesicular bodies). ($\times 13050$)

COLO 357 pancreatic cell line and its sublines), thus eliminating tumor-type specific interferences.

It is now widely accepted that many malignant tumors contain heterogeneous subpopulations of cells. This heterogeneity is manifested in a wide range of biologic, biochemical, immunologic, and genetic characteristics. It is speculated that a distinct population of tumor cells within a tumor is the forerunner of distant metastases (37). Investigation of the metastatic potential of tumor cells began in 1939 with Koch (38), who isolated a highly metastatic subline from the Ehrlich carcinoma. Since then, considerable progress has been made by many investigators, who have isolated tumor cell lines with increased metastatic potential from a wide array of

tumors, such as mouse mammary tumors (39), murine MLA-induced fibrosarcomas (40), murine sarcoma virus-transformed fibrosarcomas (41), transformed rat liver epitheloid cell lines (42), and murine B16 melanomas (43). Recently, Morikawa et al. (44) and Shishido et al. (45) implanted selected human colon and pancreatic carcinoma cells of high and low metastatic potential and concluded that the high metastatic potential cells survived in the liver to produce metastases, whereas low metastatic cells did not. Thus, surviving cells are endowed with this unique characteristic (44,45). In a previous study, we demonstrated that the human pancreatic carcinoma cell line COLO 357 is heterogeneous with regard to potential for hematogenous metastasis (46).

The two variants derived from this line, FG and SG, show distinctly different metastatic potential to the liver and lungs of the nude mouse following intrasplenic injections. The fast-growing variant FG (47) produced regional lymph node metastasis in 58% of nude mice after subcutaneous implantation and growth. The same variant produced hepatic metastases in 64% and pulmonary metastases in 43% of nude mice after intrasplenic implantation of tumor cells (46). The slow-growing variant SG did not produce any metastases after either subcutaneous or intrasplenic implantation (46,47). This heterogeneity of metastatic potential supports the hypothesis that metastasis is a nonrandom selective process (48). However, it has been proposed that metastatic dissemination occurs because of purely mechanical factors resulting from the anatomical structure of the vascular system (49). Selective isolation of tumor cells that are capable of producing nonrandom metastasis in specific organs from a single tumor has been reported (50,51); however, there are controversial reports (52,53).

In this study, increased metastatic potential is demonstrated using the human pancreatic cancer cell line COLO 357 and its derivative lines FG, L3.1, L3.2, L3.3, L3.4, and L3.5. The metastatic capacity of COLO 357 successively increased from 71 to 100% after six sequential passages through the nude mouse liver. The growth pattern of the metastatic liver deposits was interesting. Splenic inoculation of COLO 357, FG, L3.1, and L3.2 gave discrete metastatic nodules, whereas L3.3, L3.4, and L3.5 gave multiple nodules that converged toward each other and occupied a major part of the hepatic parenchyma. This progressive metastatic capacity was associated with lymph node, splenic, and pancreatic involvement (see Table 1). Parallel observations were made with the growth of subcutaneous implantations (Table 2).

Using this model, we were able to demonstrate a positive correlation between metastatic potential and cathepsin B activity. However, ultrastructural data of the tumor cell sublines used in our work did not demonstrate a similar association or any discriminating morphological features. A possible explanation may be that once the malignant nature of the cells is initiated, the phenotypic expression of cancer cells is a mainly functional property. Various factors related to tumor growth and invasion may contribute to disease progression of pancreatic carcinomas, such

as urokinase plasminogen activator (54), type IV collagenase (55), basic fibroblast growth factor (56), and transforming growth factor β isoforms (57), supporting the concept that metastatic potential is a functional quality.

In conclusion, our study established an in vivo model of graded metastatic potential for the comparison of metastatic potential of pancreatic adenocarcinoma cells and biological parameters. We found that metastatic capacity correlates well with cathepsin B activity but not with ultrastructural characteristics. Our model may be used for studying tumor cell–host interactions that mediate metastasis, testing of putative antimetastatic agents, and functional aspects of pancreatic cancer.

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