Vol. 83, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 14, 1978 Pages 144-150

EXPRESSION OF MELANOMA NEUTRAL PROTEINASE AND COLLAGENASE POTENTIAL BY ENDOCYTOSIS*

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Received April 21,1978

SUMMARY: Neutral proteinases and collagenase production were determined for four cell lines of B-16 melanoma grown in culture. Endocytosis of 1 μ latex beads by cells in vitro revealed the proteinase production potential of three cell lines. These results were correlative to the proteinases from isolated metastatic lung foci. The association of proteinase secretion to intracellular latex accumulation was best associated with a cell line with low in vitro basal proteinase activities. The role of endocytosis of material by tumor cells to metastases is discussed.

The role of proteinases in metastasis has been implied by microscopic interpretations (1,2,3) and in vitro characterization of tumor cell enzymes against collagen (3-6), basement membranes (7), and synthetic substrates (8,9). Some studies additionally suggest that the character of proteinases isolated from tumor cells grown in vitro are correlative with the invasiveness of the tumor in a host (10).

The present report demonstrates that adjunct endocytosis of material by melanoma cells in culture facilitates more complete expression of the production potential of tumor cell neutral proteinases and collagenase. These data also imply that the ingestion of material by tumor cells may be an important feature of invasion during metastasis.

MATERIALS AND METHODS: Four cell lines of B-16 mouse melanomas originally derived from solid tumor were adapted to culture (11,12). The cells were grown at 37° in Dulbecco modified media containing 10% fetal calf serum in an atmosphere of 95% air and 5% CO_2.

*Supported in part by USPHS Grant AM 15317.

0006-291X/78/0831-0144$01.00/0

Endocytosis of material by melanoma cells was accomplished by plating 10^6 cells in 75 cm^2 Falcon flasks containing 1.3^5/ml 1 μ latex beads (E. F. Fullam) in media containing serum. After 18 hours of latex incubation the cells were allowed to recover for 24 hours in media and serum. The cultures were then washed to eliminate serum and grown in serum-free media for an additional 72 hours. Media and cells were harvested by decantation, centrifugation, and physical scraping and assayed for neutral proteinases and collagenase.

Neutral proteinases were determined by using the general proteinase substrate, Azacoll (Calbiochem.), after the method of Werb and Reynolds (13). Collagenase activities were assayed after activation of latent enzyme with trypsin and inhibition with soybean trypsin inhibitor (14). Collagenase substrates were prepared by the method of Hashimoto et al. (3) and collagenase determined (15) by using 1,10-phenathroline and cystiene as inhibitors of neutral proteinase and collagenase, respectively.

The degree of latex bead endocytosis for each cell line was determined by counting individual beads in 100 tumor cells per flask with an Invertoscope D (Zeiss, Oberkochin, DBR) and a X40 water immersion objective.

Pulmonary metastases were induced by harvesting tumor cells from culture and injection (10^5 cell/.1 ml) into the tail vein of a C57BL/6J host mouse (16). Metastatic tumors were then isolated and individual tumors from a single host were pooled for proteinase and collagenase determination and/or reestablished into culture.

RESULTS AND DISCUSSION: Initial characterization of the four melanoma cell lines revealed that all four cell lines produced neutral proteinases to a variable degree (Table I). Three of the cell lines secreted collagenase into the culture medium (Table I). Treatment of the cells with 1 μ latex beads resulted in endocytosis of beads and an increase in the level of neutral proteinases and collagenases in three of the cell lines, one (OB-16) which did not initially produce collagenase (Table I). The remaining cell line (Amel) was refractive and did not produce any additional proteinases after latex ingestion (Table I).

These data on tumor cells are in consort with the observation made on fibroblasts (13,17), indicating that optimal thresholds of neutral proteinase and collagenase were obtainable after the incorporation of a fixed number of latex particles. The refractiveness of Amel cell line indicates that these potentials were achieved prior to endocytosis of latex.

Characterization of isolated metastatic tumor foci showed that both neutral proteinases and collagenase were present, though not to a similar

Table I

The effect of latex bead ingestion on neutral proteinase and collagenase secretion by four cell lines of B-16 melanoma

Tumor Cell Line*	Harvested Cells units/mg protein/72 hours				Culture Medium units/flask/72 hours			
	CONTROL		LATEX		CONTROL		LATEX	
	Collagenase	Neutral Proteinase	Collagenase	Neutral Proteinase	Collagenase	Neutral Proteinase	Collagenase	Neutral Proteinase
Amel	.117 ±.003	.060 ±.010	.121 ±.010	.075 ±.009	1.23 ±.04	.930 ±.010	1.21 ±.05	.993 ±.030
RB-16	.082 ±.002	.054 ±.009	.161 ±.011	.075 ±.005	.98 ±.02	.723 ±.061	2.14 ±.03	.876 ±.081
OB-16	0	0	.141 ±.020	.026 ±.001	0	.036 ±.009	1.34 ±.06	1.143 ±.142
MFH	.066 ±.011	.048 ±.002	.188 ±.022	.066 ±.001	.71 ±.01	.723 ±.050	2.13 ±.02	1.056 ±.081

*Designation of tumor cell lines are only laboratory references. One unit of collagenase activity was defined as the enzyme activity which will hydrolize 1 μg of collagen gel/min at 36.5°C in a Tris/NaCl/$CaCl_2$ (15) buffer. One unit of neutral proteinase activity is defined as the hydrolysis of 1 mg of Azocoll per hour at 37°C read at 520 nm on a Beckman spectrophotometer in a Tris/NaCl/$CaCl_2$ buffer (13). Data represent the mean of five experiments.

Table II

Collagenase and neutral proteinase activities
from isolated metastatic tumors

Cell Line	Collagenase (units/mg Protein)	Neutral Proteinase (units/mg Protein)
Amel	.332 ±.015	.141 ±.011
RB-16	.416 ±.021	.099 ±.021
OB-16	.356 ±.041	.162 ±.032
MFH	.221 ±.061	.058 ±.044

Collagenase and neutral proteinase activities were defined in Table I. Data represent the mean of three experiments.

degree in each tumor cell line (Table II). Readaption of the isolated metastatic tumors to culture resulted in a proteinase phenotype similar to the original culture inoculum used to establish metastasis (Table III).

Although the failure to demonstrate collagenase in culture subsequent to its measurement in isolated metastatic foci (5) suggests an extratumoral enzyme represented by either monocyte or neutrophil contributions, the present report indicates that the activity of tumor cell collagenase may in some cell lines be susceptible to a variable expression. Thus, the absence of collagenase subsequent to in vitro growth does not always indicate the potential of that proteinase production of the tumor. These data also suggest that ingestion of material by melanoma cells can be an important feature of collagenase and neutral proteinase production and possibly influence tumor cell invasion and metastasis. The demonstration of an enhancement of metastasis by plastic spheres and organic debris incorporated into the inoculum of metastasis experiments (18) may be interpreted to support the later speculation.

In this study the amount of latex beads ingested were not generally

Table III

Collagenase and neutral proteinase activities secreted into media by four cell lines readapted to culture from metastatic tumors

Cell Line	Collagenase	Neutral Proteinase
Amel	.98 ±.01	.721 ±.020
RB-16	.79 ±.04	.551 ±.021
OB-16	0	.012 ±.011
MFH	1.41 ±.03	.861 ±.032

Collagenase and neutral proteinase activities defined in Table I. Data represent mean of three experiments. Activity is total units/flask/72 hours, culture medium free of serum.

correlative to the amount of neutral proteinase or collagenase secreted in vitro (Table IV). However, the OB-16 cell line characterized by low basal neutral proteinase and the absence of collagenase did show such a relationship (Table IV). Werb (17,19) has suggested that the amount of intracellular membrane and plasma membrane turnover are related to endocytosis of materials and the secretion of extracellular proteinases. The present study indicates that this relationship may only be applicable to cell lines with low basal proteinase levels.

Recognizing that the degree of latex bead accumulation in the cell lines Amel, MFH, and RB-16 were drastically different with similar proteinase production, we speculate that the level of endocytosis subsequent to the achievement of optimal protease production is determined by other factors, such as the biochemical character of the plasma membranes which determines their stickiness. Although increased metastatic capabilities of tumor cells have been correlated with an increased sialic acid content of their cell surfaces (20), Fidler (21) has noted that this observation may not always be applicable.

Table IV

Relation of latex bead endocytoses to level of neutral proteinase secretion

Cell Line	Latex beads/cell	Neutral Proteinase units/flask/72 hours
Amel	191.66 13.84	.993 .030
RB-16	16.00 4.00	.876 .081
MFH	31.60 5.62	1.056 .081
OB-16	71.25 8.44	1.143 .142
OB-16*	31.82 4.65	.862 .061

Data represent the mean of four experiments.

*Reduced beads in OB-16 were obtained by seeding cultures with $6 \cdot 5^4$ beads/ml.

In other cell lines $6 \cdot 5^4$ beads/ml were optimum for proteinase secretion.

The role of endocytosis in controlling the secretion of non-lysosomal proteinases has been well documented in matrix degradations (17). The present investigation has shown that this may be an ubiquitous phenomenon or, at least, partially extended to include a nonconnective tissue tumor cell system.

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