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Glycosaminoglycan-sac formation in vitro. Interactions between normal and malignant cells

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Summary. The interaction of monolayer normal human or normal rat cells with suspension Walker rat tumor cells was demonstrated cytologically, during a co-cultivation period of thirty days. At ten days, Walker rat tumor cells were interiorized in the cytoplasm of the normal monolayer host cells. At twenty days, degeneration of the interiorized tumor cells followed by mucification led to glycosaminoglycan-sac formation. At thirty days, tumor nodules and protease (α_1-chymotrypsin)- bound glycosaminoglycan(s) extracellular matrix, transversing the culture in membranous rivulets, were formed. The overall interaction resulted in the death of the interiorized tumour cell and survival of the normal host cells containing a glycosaminoglycan-sac. The use of bladder tumor cell imprints showed that cell interiorization, degeneration and extracellular matrix formation identical to that of the co-cultures, also occur in vivo. Cell interiorization within malignant cell cultures led to syncytia formation and survival of both host and interiorized cells. Identical glycosaminoglycan extracellular matrix to that of the co-cultures and syncytia was also observed in smears of EBV-producing (P3HR-1) cell cultures. The observations are discussed in terms of invasion, emperipolesis, cannibalism, phagocytosis and extracellular matrix. It is concluded that a glycosaminoglycan-sac is produced by the interiorization of a tumor cell into a normal cell while interiorization of a tumor cell into another tumor cell resulted in syncytia formation.

Key words: Emperipolesis, Cannibalism, Extracellular matrix, Interaction, Normal with malignant cell cultures, Protease, Glycosaminoglycans, Invasion

Introduction

Cytology of malignant and embryonic cell cultures had revealed intracellular production of green-translucent glycosaminoglycans (GSG), enclosed in a membranous sac (GSG-sac) (Logothetou-Rella et al., 1988, 1989). These GSG sacs pass extracellularly and form a solid green-fibrillar-translucent extracellular matrix (ECM) which covers the cells, enhances tumor nodule (Logothetou-Rella et al., 1992a) and nuclear vlimata formation (Logothetou-Rella, 1993a).

This ECM was produced abundantly by lung cell cultures consisting of both normal and malignant cells (Logothetou-Rella et al., 1992a).

Also, GSG-sacs were identified in vivo, in mechanically-dispersed bladder tumor cells associated with protease(s) (Logothetou-Rella et al., 1992b).

Furthermore, lymphocytes, upon contact with the GSG-protease-membrane ECM, produced nuclear vlimata and changed the fusiform-type tumor nodule into the adenoid type (Logothetou-Rella, 1993b).

In the development of new anticancer agents, this solid ECM should be taken into consideration, as it may act as a permeability barrier and inhibit access of cells to the anticancer agent.

It is therefore necessary to investigate the origin and production of GSG-sac in vitro and in vivo.

In the present study an attempt is made to produce GSG-sacs in vitro, using co-cultures of normal and malignant cells.

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Materials and methods

Cell cultures - Cytology

Wistar rat normal skin cell culture was initiated from skin biopsy. The biopsy was sliced into small pieces, rinsed with complete medium RPMI-1640 and placed on the surface of a 25 cm^2 tissue culture flask (Costar) with fine forceps. The flask with the tissue pieces was incubated upright for 45 min and then 3 ml complete medium RPMI-1640 was added. The medium was changed every seven days and the culture flask surface was covered with cells in one month. The cells were then subcultivated by conventional trypsinization.

Also monolayer normal fallopian tube F-cells (Logothetou-Rella, 1993b), melanoma Ha-cells, malignant bladder Pa-cells, lung (malignant with normal) M-cells (Logothetou-Rella, 1993a), suspension Walker rat tumor cells and Burkitt lymphoma EBV-producing P3HR-1 cell cultures (donated by Dr. S. Kottaridis) were used for cytological studies.

All cell cultures were grown in complete medium RPMI-1640 (Gibco), supplemented with 10% foetal bovine serum (Gibco), penicillin (100 U/ml, Gibco), and streptomycin (100 µg/ml, Gibco) and incubated at 37 °C in a CO_2-humidified incubator. Stock cells were stored frozen in liquid nitrogen.

For cytology, the monolayer cell cultures grown in glass petri dishes and smears of the suspension cell cultures were fixed in 50% ethanol and stained by Papanicolaou, PAS and PAS-diastase.

Co-cultivation of normal with malignant cells

Suspension Walker rat tumor cells were added to confluent monolayer F-cells or Wistar rat skin cells, and co-cultivated in glass petri dishes containing 10 ml complete medium. Twelve petri dishes with co-cultures were set up in each case. The co-cultures were allowed to interact by incubation at 37 °C in a CO_2-humidified incubator for 30 days of continuous co-cultivation. The co-culture medium was changed every three days and rinsed many times by gently pipetting medium on top of the culture, in order to remove the overpopulated Walker rat tumor cells. At intervals of ten, twenty and thirty days of co-cultivation, the co-cultures and control cell cultures were fixed in 50% ethanol and stained by Papanicolaou, PAS and PAS-diastase.

Thirty-day-old co-cultures were subcultivated by trypsinization and after seven days of growth were used for cytology.

Immunocytochemistry

Wistar rat skin cells, F-cells, the co-cultures and cell imprints from five patients with invasive bladder carcinoma were fixed in cold acetone (-20 °C) for 10 min and used for immunocytochemical studies. The avidin-biotin peroxidase complex method was applied (Hsu et al., 1981) using the antisera against α_1-chymotrypsin (1:150, A022, Dako Corp.) and macrophage antigen (1:40, M718, Dako Corp.). Positive controls and negative controls by omitting the primary antiserum were used.

Electron microscopy

Pellets of trypsinized F-cells, Wistar skin cells, and the co-cultures were fixed in phosphate-buffered 4% glutaraldehyde, postfixed in buffered 1% osmium tetroxide, dehydrated in graded ethanols and embedded in Epon 812. Thin sections for electron microscopy were stained with uranyl acetate and lead citrate.

The co-cultures were also fixed directly by addition of phosphate-buffered 4% glutaraldehyde and were observed under the phase contrast microscope.

Results

Cytology of the co-cultures

Normal F and Wistar rat skin cells consisted of epithelial and stromal cells growing in monolayer. Walker rat tumor suspension cells were easily recognisable in the co-cultures by their round morphology, hyperchromatic, pyknotic nucleus and very little cytoplasm. After ten days of co-cultivation, cytology showed that normal monolayer F-cells or Wistar rat skin cells interiorized the free Walker tumor cells. Each normal cell interiorized one to ten Walker

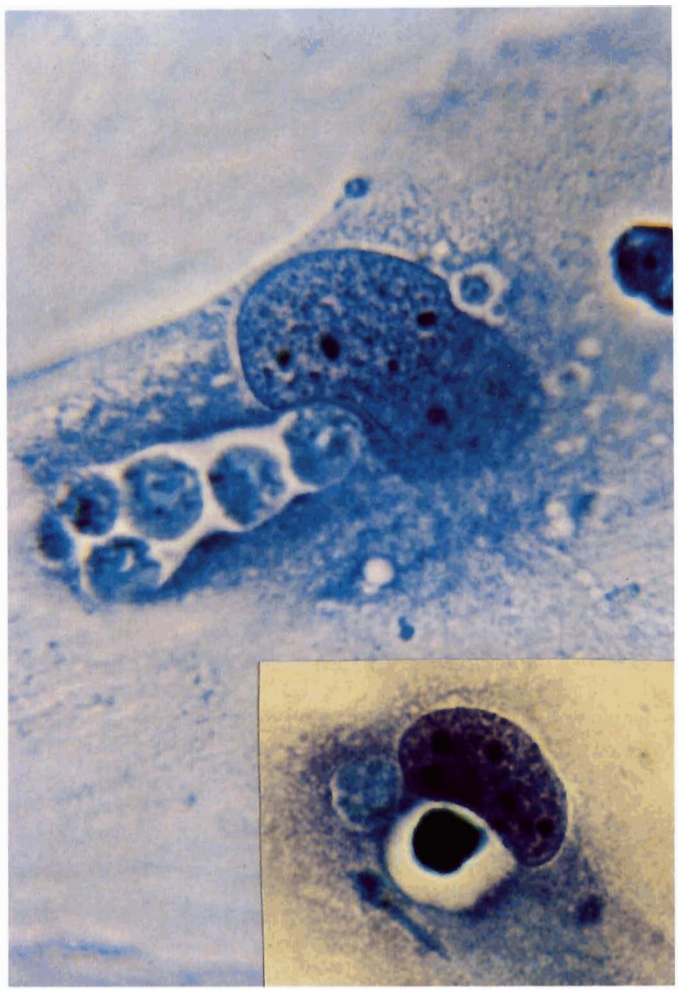

Fig. 1. Five interiorized Walker rat tumor cells in a normal Wistar rat skin cell. Degeneration of the interiorized cells is characterized by the particulate green texture and nuclear remnants. Papanicolaou. x 1,000 phase-contrast. Inset: two interiorized cells; one degenerating of light green colour and one after mucification in the form of GSG-sac, of dark green translucent texture. Papanicolaou. x 1,000

tumor cells. Each of the intracellular Walker tumor cells was observed either intact or with a different degree of degeneration, exhibiting light green particulate texture by Papanicolaou stain (Fig. 1).

After twenty days of co-cultivation most of the interiorized Walker tumor cells were completely degenerated giving rise to a round, translucent, dark green, well-defined membranous sac, surrounded by a clear halo (Fig. 1 inset).

After thirty days of co-cultivation the intracellular green sacs were devoid of cellular elements, or contained intact Walker tumor cell nuclei or nuclear remnants (Fig. 2). Cell nodules of mixed normal and Walker tumor cells were observed. The membrane extensions of the intracellular green sacs transversed through the normal monolayer cells forming rivulets (Fig. 3) which entered the cytoplasm of neighbouring cells (Fig. 3 inset). The interiorized cells, the translucent content of the membranous sacs and rivulets was PAS and PAS-diastase positive, which confirmed the presence of GSG (Fig. 4). Cytology of the subcultivated co-cultures showed survival of the GSG-sac containing host cells and maintenance of the ECM. Control F or Wistar rat skin cells contained cytoplasmic fine, PAS- and PAS-diastase-positive granules and did not develop GSG-sacs.

Immunocytochemistry

Fallopian tube F-cells and Wistar rat skin cells showed negative immunoreactivity for macrophage antigen.

Cell nodules and GSG-sacs in the co-cultures showed positive immunoreactivity for α_1-chymotrypsin, while monolayer host cells were negative. Walker rat tumor cells in round form and nuclear vlimata showed positive immunoreactivity for α_1-chymotrypsin, of different

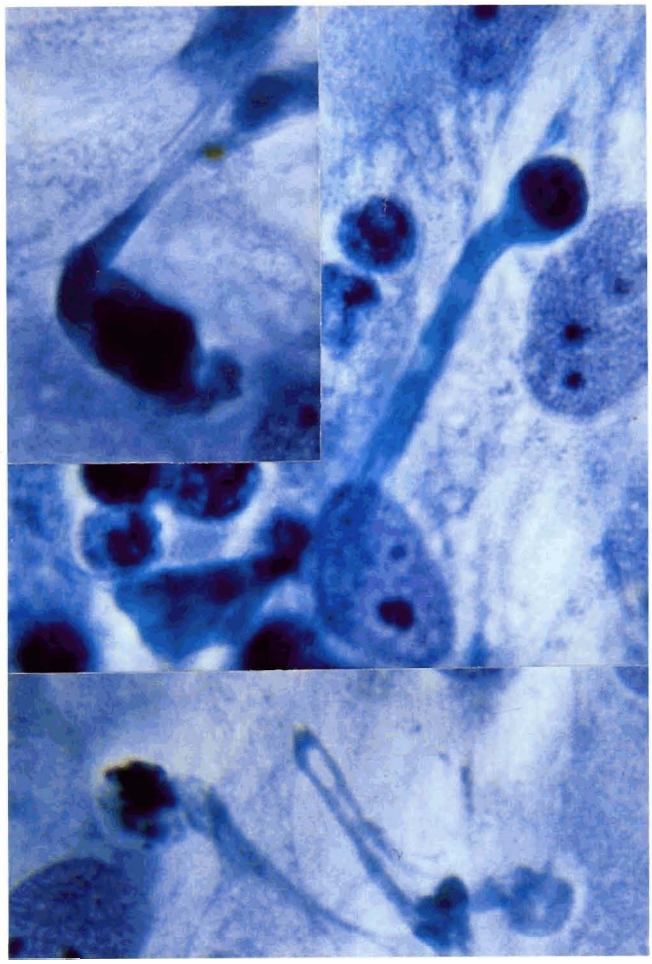

Fig. 2. Thirty-day-old co-cultures of F-cells with Walker rat tumor cells. Intracellular GSG-sacs, containing intact nucleus or nuclear remnants of interiorized Walker rat tumor cells, have developed membrane extensions extracellularly. Papanicolaou. x 1,000

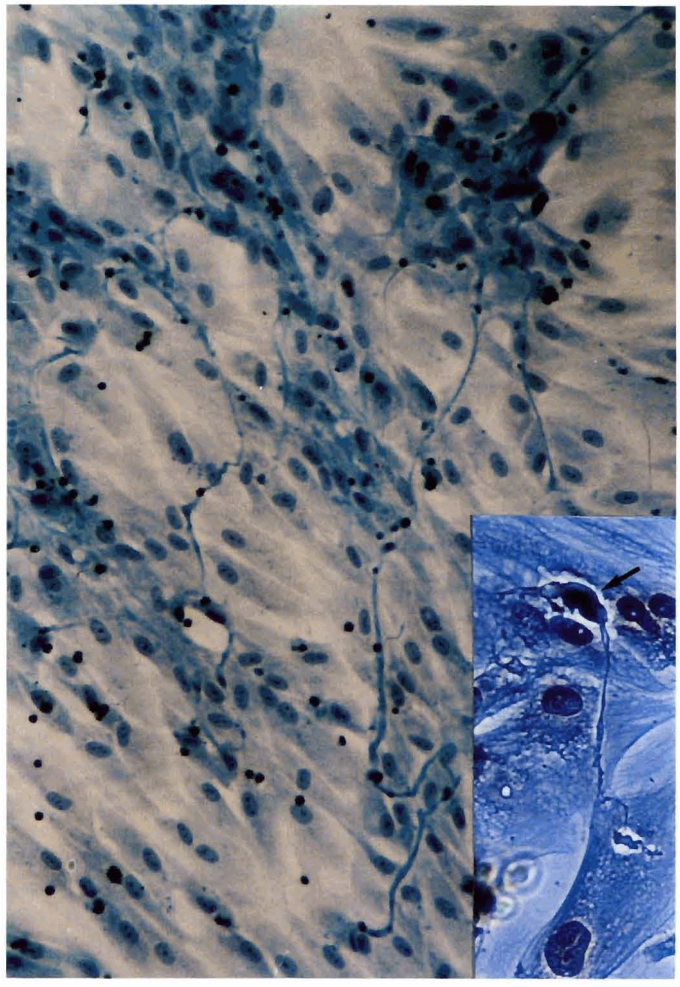

Fig. 3. Thirty-day-old co-culture of normal F-cells with [redacted] rat tumor cells. Membranous green rivultes full of GSG are transversing the monolayer F-cells. Papanicolaou. x 100. Inset: A GSG-sac (arrow) giving rise to rivulets, the content of which invades the cytoplasm of a neighbouring cell. Papanicolaou. x 200 phase contrast.

intensity (Fig. 5).

Urothelial cell imprints from invasive bladder carcinoma showed interiorized degenerating cells with strong immunoreactivity for α_1-chymotrypsin (Fig. 6).

Cytology of cell cultures

Cytology of the different cell cultures was focused on the presence of interiorized cells, syncytia and GSG ECM.

Melanoma Ha-cell culture and malignant bladder Pa-cell culture showed cells which interiorized each other leading to large syncytia formation (Fig. 7). No GSG-sacs or tumor nodules were observed in these pure malignant cell cultures.

The M-cell culture (normal and malignat cells) previously characterized (Logothetou-Rella et al., 1992a), besides the presence of GSG-sacs and tumor nodules, exhibited small interiorizing cells forming cytoplasmic junctions with larger, attached host cells and subsequent interiorization (Fig. 8).

Urothelial cell imprints from invasive bladder carcinoma showed green, particulate, translucent ECM and degenerated interiorized cells of green translucent texture with nuclear remnants (Fig. 9).

Smears of P3HR-1 (EBV-producing) cells showed syncytia, nuclear vlimata, binucleated and mononucleated cells embedded in and around accumulated green GSG, which was PAS- and PAS-diastase-positive (Fig. 10).

Electron microscopy

No difference was detected in lysosomes, rough endoplasmic reticulum and Golgi apparatus between F-cells in the control and F-cells in the co-cultures, at the ultrastructural level.

Direct fixation of the co-cultures removed the ECM

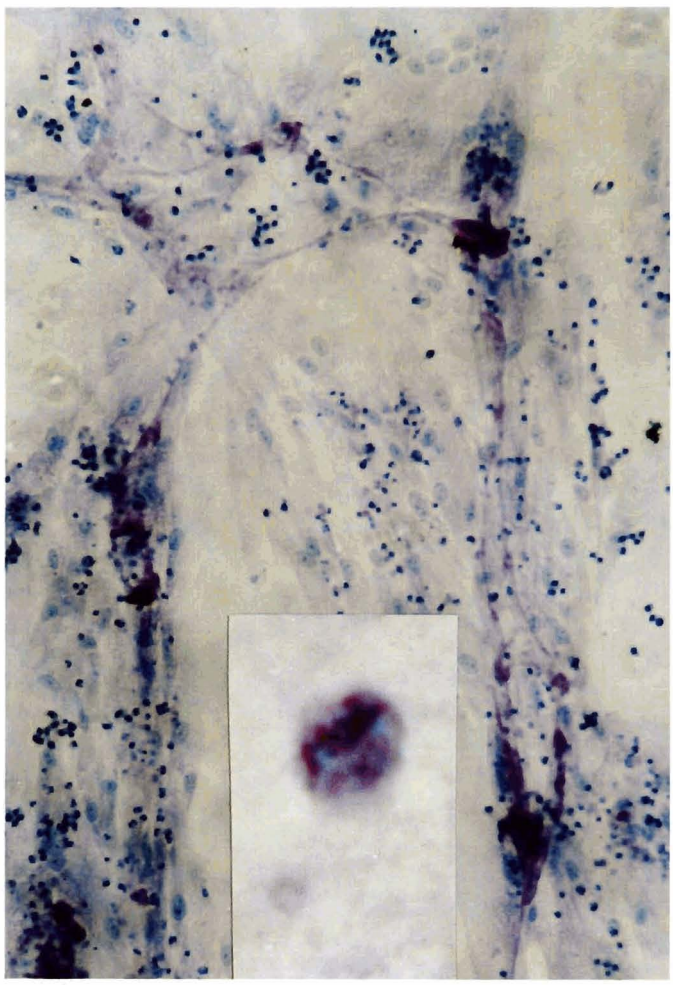

Fig. 4. As in Fig. 3 PAS-Diastase. x 100. Inset: Degenerating, mucifying interiorized Walker rat tumor cell. PAS-diastase. x 1,000

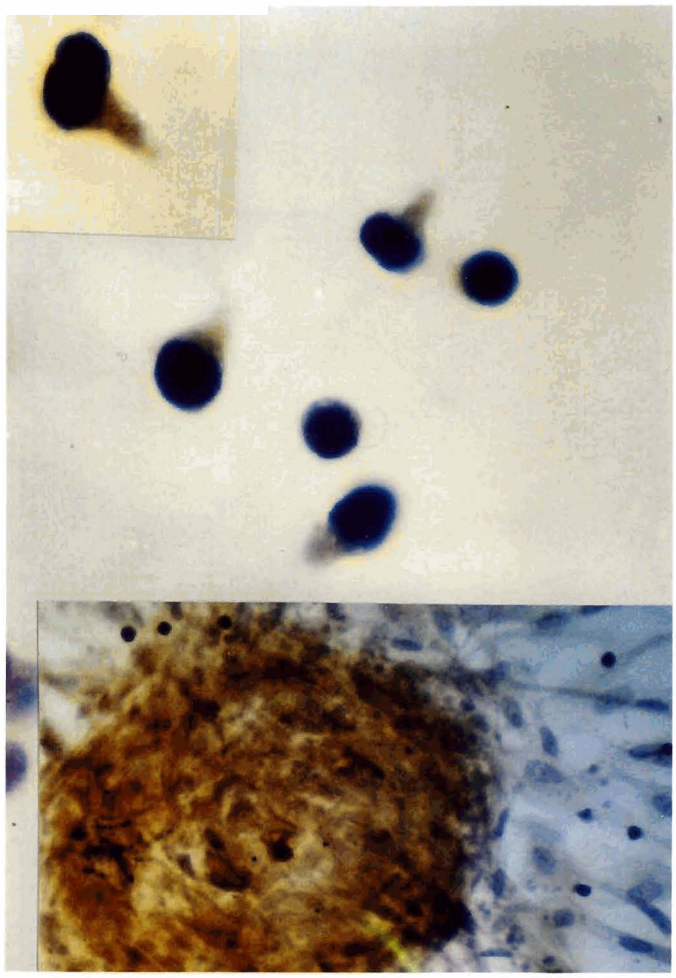

Fig. 5. Walker rat tumor cells in the form of nuclear vlimata, positive for α_1-chymotrypsin showing different intensity of immunoreactivity. α_1-chymotrypsin. x 1,000. Inset: Tumor nodule positive for α_1-anti-chymotrypsin in 30-day-old co-cultures. Monolayer host cells are negative. α_1-antihymotrypsin. x 100

from the cells, in a large, jelly-like sheet floating in the fixation solution.

Discussion

Interaction studies of normal with malignant cells have been performed in the past but the co-cultivation period was limited to seventy-two hours (Brouwer et al., 1984; Delinassios and Kottaridis, 1984). In the present study the co-cultivation period was extended to thirty days, for neoplasia is considered as a long time process and tumors are constituted by normal and malignant cells.

The results showed that malignant cells interiorize in other cells. This cell interiorization leads to different cellular events and products which depend upon the state of the host cell. Malignant cell interiorization into malignant cells (Pa and Ha-cells) leads to syncytia formation and survival of both host and interiorized cell.

Malignant cell interiorization into normal cells (co-cultures, M-cells) leads to death of the interiorized cell, survival, propagation of the host cell and production of protease-bound GSG-sac and ECM. Intracellular degeneration of the malignant interiorized cell is accompanied by mucification and protease production (positive for α_1-chymotrypsin), in vitro and in vivo, which does not affect survival and propagation of the host cells. The ECM produced by such cell interaction enhances tumor nodule and nuclear vlimata formation (Logothetou-Rella, 1993a).

In addition, Walker rat tumor cells in the form of nuclear vlimata possessed proteases (positive for α_1-chymotrypsin) enabling the invasion of other cells.

The mechanism of the intracellular degeneration of the interiorized malignant cell is unknown. It could be initiated by the lack of nutrition and subsequent autolysis and mucification by its intracellular proteases and/or by the host cell intracellular proteases other than lysosomal enzymes.

The amazing phenomenon of this long term cell interaction is the survival of host cells. The GSG-sac is

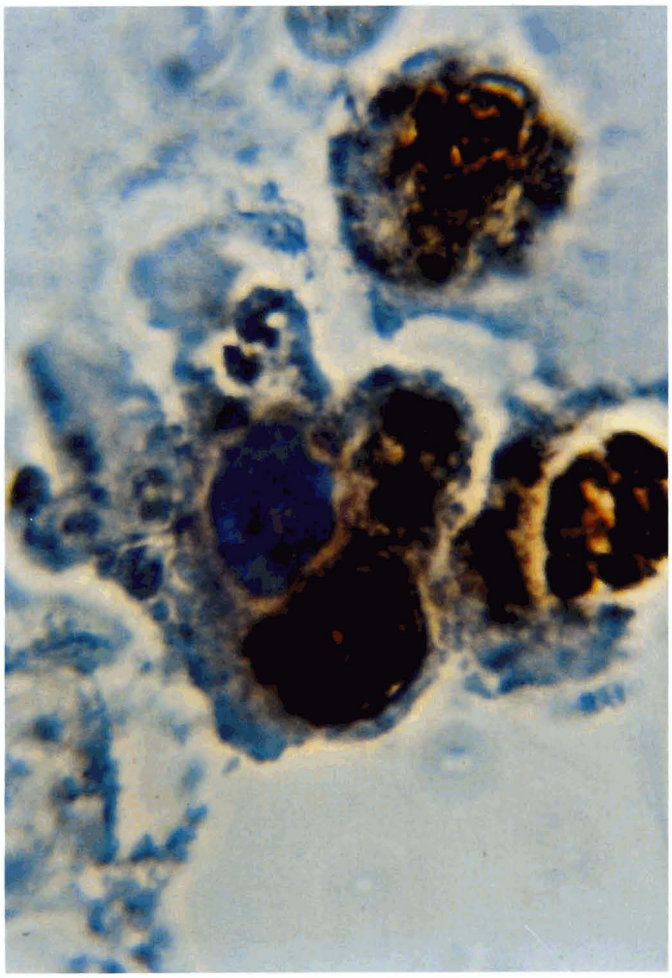

Fig. 6. Cell imprints from invasive bladder carcinoma. Degenerating interiorized cell is positive for α_1-chymotrypsin. α_1-antichymotrypsin. x 1,000

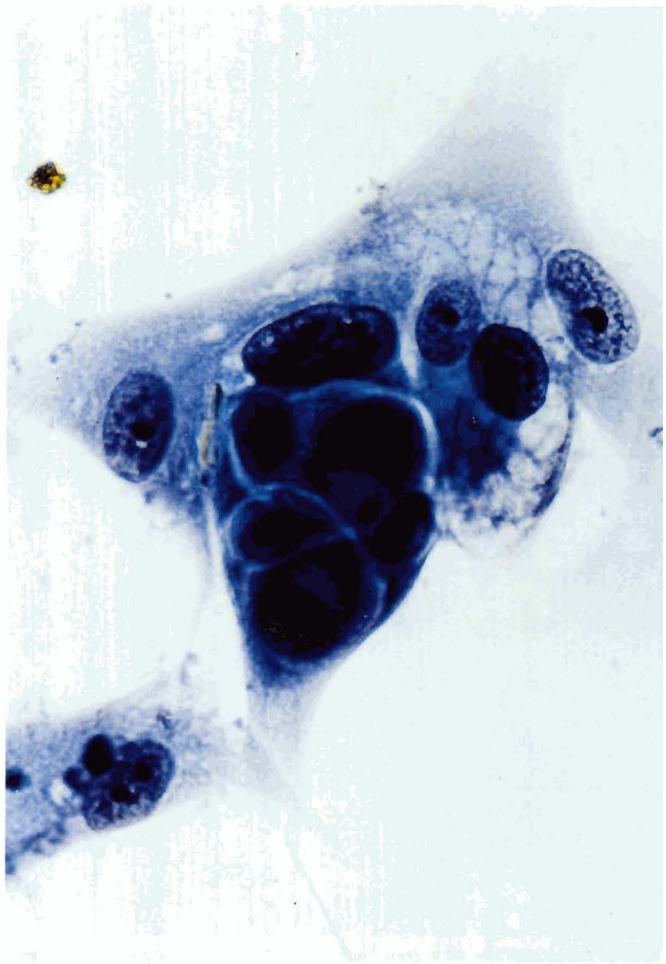

Fig. 7. Large syncytia formation by melanoma Ha-cells. Papanicolaou. x 400

membranous, surrounded by a halo, thus preventing contact of the sac contents with the host cell interior. Moreover, the protrusion of membrane extensions extracellularly, in the form of membranous rivulets, indicates that GSG-sac formation is the means by which host cells expel the waste products of such interaction. It appears this is a defence mechanism of host cells against invader cells.

The GSG-protease ECM is dissolved and detached upon fixation of the co-cultures for ultrastructural study. It has therefore been difficult to characterize it at the ultrastructural level. Previous study has shown the existence of enlarged modified lysosomes in bladder tumor cells well correlated with the GSG-sacs (Logothetou-Rella et al., 1992b). The lack of differences in the lysosomes of host cells (free of macrophages) in the control cell cultures and in the co-cultures, indicates that cell interiorization, degeneration and mucification do not follow the process of conventional phagocytosis.

The ECM produced by cell interiorization was observed invading and being deposited in the cytoplasm of a neighbouring cell, free of cell interiorization. This observation, already reported and interpreted (Logothetou-Rella et al., 1988), strengthens the protease association with GSG and the existence of GSG-sacs in cells free of cell interiorization.

Although it has been reported that HIV-infected T-cells form syncytia in vitro (Marx, 1991), there has been no cytological examination of the ECM formed by these cells. In this study cytology of virus-producing cells (P3HR-1) showed that besides syncytia and nuclear vlimata, these cells also produce ECM identical to the one formed by the co-cultures.

The event of «tumor cell within a tumor cell» has frequently been recognised in vivo and in vitro. Ehrlich ascites tumor (EAT) cell with another EAT cell has been observed in vitro, and the process characterized as emperipolesis (Burns, 1967). Ultrastructural study of emperipolesis of tumor cells, in vivo, did not show degeneration of the interiorized or host tumor cells (Chemnitz and Bichel, 1973). Furthermore, in cell lines

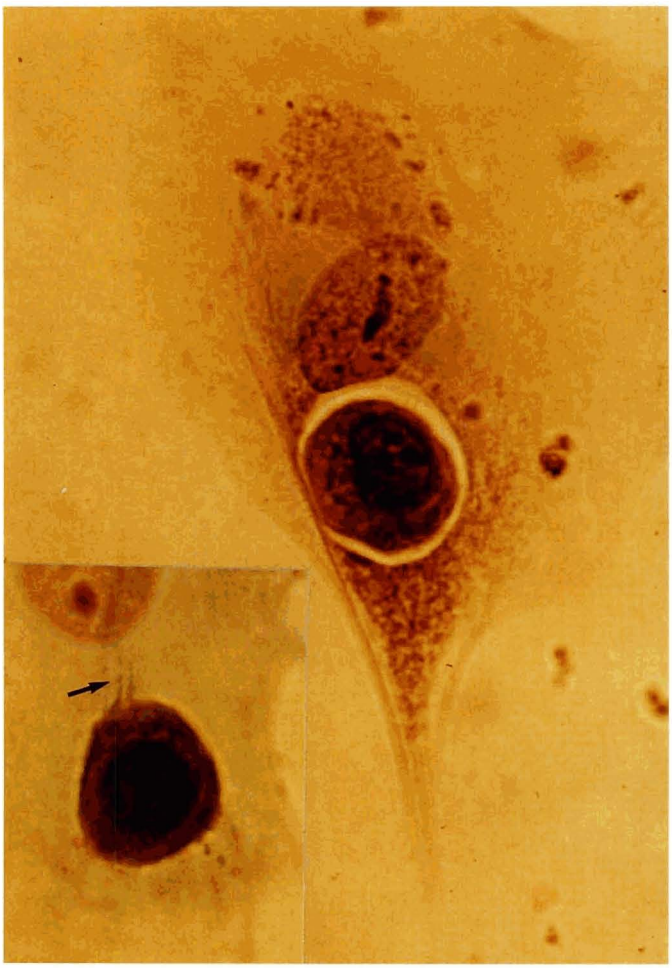

Fig. 8. Interiorization of a small cell into a large attached cell in M-cell cultures. Papanicolaou. x 1,000. Inset: Cytoplasmic junctions (arrow) of the small cell can be observed just before interiorization. Papanicolaou. x 1,000

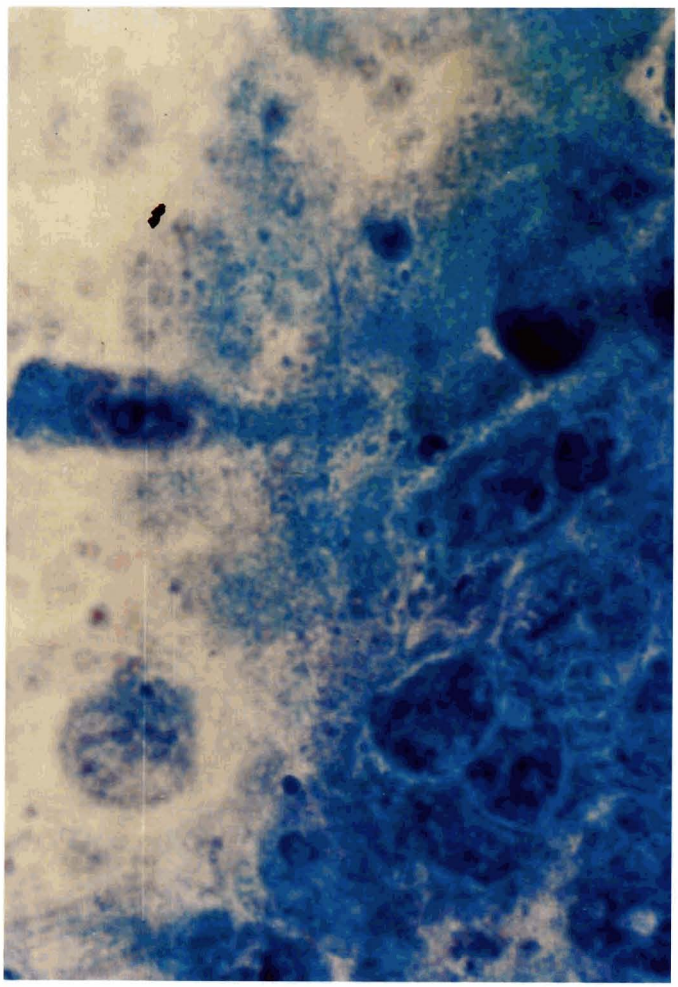

Fig. 9. Cell imprints form invasive bladder carcinoma showing green degenerating interiorized cells and ECM. Papanicolaou. x 1,000

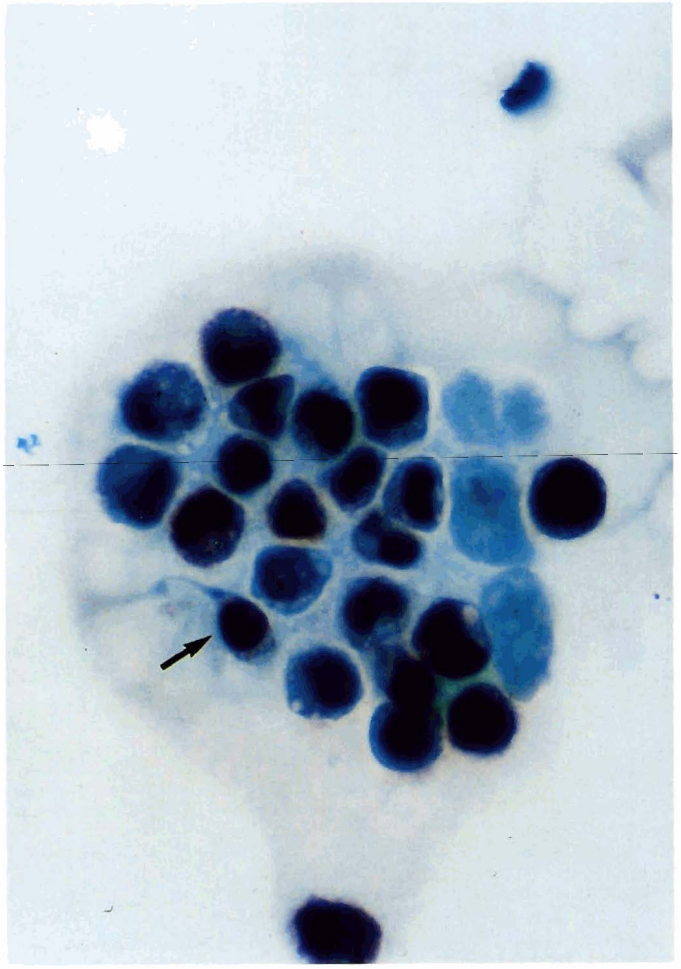

Fig. 10. Smears of P3HR-1 (EBV-producing) cells showing accumulated green ECM. Arrow points at a nuclear vlima. Papanicolaou. x 1,000

of human small carcinoma, malignant cells interiorized other malignant cells leading to complete auto-destruction of the cultures. This process has been characterized as cannibalism (Brouwer et al., 1984). In addition, interiorized tumor cells have been observed as functional and characterized parasitic as they were dividing inside the host cells, producing nuclear vlimata directed extracellularly (Logothetou-Rella, 1993a). In the present study the interiorized malignant cell is degenerated and mucified inside a normal cell, giving rise to a specific ECM. All these processes have been observed in vitro and in vivo.

The available literature information and the present observations on cell interiorization tend to agree that malignant cells reach an unstable phase at which they seek a host cell support, at random, for survival and propagation. Cell interiorization can be achieved by invasion, via cytoplasmic jucntions and proteases exhibited by malignant cells. Subsequent fate of the interiotized tumor cells may depend on both host and interiorized cell state, until now unknown.

In conclusion, in this study, GSG-sacs and specific ECM were produced in vitro by the interaction of malignant with normal cells, without excluding other means of production. It is suggested that this ECM should be considered in the development of new anticancer agents.

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