Differential Expression of Intermediate Filament Proteins in Metastatic and Nonmetastatic Variants of the BSp73 Tumor

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ABSTRACT

The pattern of intermediate filament protein expression was studied in tumor cell variants of the BSp73 spontaneous rat adenocarcinoma of the pancreas exhibiting distinct morphology and metastatic phenotype. The non-metastasizing AS cells which adhere and spread on a solid substrate express only the vimentin type mesenchymal intermediate filament protein. The ASML metastatic cell variant which adheres but does not spread on the substrate expresses a complex pattern of cytokeratins characteristic of the adenocarcinoma of the pancreas and a low level of vimentin. The differences in the expression of the intermediate filament proteins between the variants were also reflected at the level of the corresponding mRNAs as revealed by RNA blot analysis with complementary DNA clones specific to vimentin and the acidic as well as the basic cytokeratin subfamily. When the two cell variants were cultured for 72 h in suspension culture on nonadhesive substrata the AS cells responded with a marked reduction in their vimentin synthesis. The ASML variant cells which are characterized by a round configuration in both monolayer and suspension culture continue to synthesize the same intermediate filament proteins under both culture conditions. The relationships among environmental conditions that affect cell shape and contacts, the shifts in the expression of intermediate filaments, and the metastatic property of tumor cells are discussed.

INTRODUCTION

Numerous studies suggest that the regulation of a variety of growth-related cellular activities are mediated through changes in cell shape (1-4; for reviews, see Refs. 5 and 6). In line with these suggestions are several recent reports which identified specific transient alterations in the organization of cytoskeletal elements in the response of the cells to purified growth factors (7, 8). While a linkage between growth control and the organization of cytoskeletal elements was suggested, the progressive loss of cell shape-responsive metabolic controls in several transformed cells was proposed to be related to the phenomenon of tumor progression (9). Furthermore, it was demonstrated that the expression of the neoplastic phenotype in C3H/10T1/2 cells (10) and the expression of the malignant metastatic phenotype in B16 melanoma could be modulated by culture conditions that alter cell contacts and shape (11). Our previous studies have shown that stable (prolonged) alterations in the organization of cytoskeletal elements are accompanied by responses at the level of expression of the corresponding cytoskeletal protein gene(s) (12-16; for review, see Ref. 5).

Recently we have analyzed the response of tumor cell variants exhibiting distinct metastatic capabilities to changes in cell shape and were able to demonstrate a reversible modulation of the metastatic lung colonizing potential by culture conditions which introduced changes in cell shape (11). Variations in cell shape that correlated with alterations in the metastatic capability were accompanied by changes in the expression of the intermediate filament protein vimentin (11, 12). In addition, by specifically disrupting the vimentin network with cycloheximide (17) in B16 melanoma we observed simultaneous changes in the metastatic capability of the cells and in the biosynthesis of vimentin (18). Furthermore, by inhibiting the polymerization of vimentin in B16 cells by canavanine in culture, a reduction in their lung colonizing in vivo was observed (19).

The recently established tumor cell variants of a spontaneous adenocarcinoma of the pancreas in BD X rats (BSp73) was of special interest because these variants display in a stable fashion several characteristics which we obtained in the B16 melanoma system by culture conditions that modulated cell shape (20). In addition, the BSp73 system brings to the extreme our previous observations on an association between the degree of cell spreading on a substrate and the metastatic capability of cells (21-23). By s.c. transplantation of the BSp73 nonmetastasizing (AS) and metastasizing (ASML) cells, variants were obtained which maintained a stable phenotype after in vitro recloning (20). The nonmetastasizing variant cells attach and spread on the culture dish, whereas the ASML cells which spontaneously metastasize to the lungs via the lymphatic vessels adhere but fail to spread on the culture dish and grow as spherical cells (20).

Several cellular properties which could be related to the metastatic capability of these variants were identified, such as resistance of the metastatic ASML variant to natural cytotoxicity by natural killer-like effector cells and macrophages (24) and a high concentration of a serine protease (cathepsin B-like) activity characteristic of metastatic cells (25). Cytogenetic analysis indicated that the metastasizing ASML cells have a higher mean chromosome number than the nonmetastasizing AS cells, but both cell types displayed similar abnormalities in several chromosomes and the loss of specific chromosomes including the sex chromosomes (26, 27).

In the present investigation we studied the pattern of intermediate filaments in the metastasizing and nonmetastasizing variants and addressed the possible relationships between cell...
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RESULTS

Two-Dimensional Gel Electrophoretic Characterization of Intermediate Filament Proteins in the AS and ASML Variants. In order to examine the intermediate filament protein pattern of the AS and ASML cell variants, cells were labeled overnight with \[^{[35}S\]methionine, and a Triton X-100 and high-salt insoluble fraction which was enriched in intermediate filaments was prepared from both cell types. Two-dimensional gel electrophoresis of these fractions revealed that the AS cells which show a well spread morphology on plastic (Fig. 1A) expresses mainly vimentin and a few characteristic proteolytic fragments of vimentin (Fig. 1C). In contrast, the adherent but spherical ASML variant (Fig. 1B) expresses a complex pattern of cytokeratins (Fig. 1D) including cytokeratins 7, 8, and 17-19 [according to the nomenclature of Moll et al. (35)]. The expression of vimentin in the spherical ASML cells was much lower in comparison to the AS variant cells. The pattern of cytokeratins obtained by the two-dimensional gel electrophoretic analysis of the ASML cells corresponds to that reported for the adenocarcinoma of the pancreas of human origin (35, 36) and thus supports the histological classification of the spontaneous tumor from which the variants were derived as adenocarcinoma of the pancreas. While the proteins of the Triton-high salt insoluble fractions show marked differences between the variants, the Triton soluble fractions (Fig. 1, E and F) that contain about 80% of the total cellular proteins show a high degree of similarity between the AS (Fig. 1E) and the ASML (Fig. 1F) cells including similar levels of actin and tubulin.

Immunoblot Identification of Intermediate Filaments in the Tumor Cell Variants. Coomassie blue staining of SDS polyacrylamide gels of the Triton-high salt cytoskeletons from the same number of AS (Fig. 2A) and ASML (Fig. 2B) cells showed a polypeptide pattern characteristic of these cells as revealed by the two-dimensional electrophoresis of \[^{[35}S\]methionine-labeled cells (Fig. 1, C and D). Using a monoclonal antibody against vimentin in the immunoblot assay with proteins identically separated but stained with Coomassie blue as shown in Fig. 2, A and B, we found that AS cells indeed express a high level of vimentin (Fig. 2C) while ASML cells (Fig. 2D) express much lower levels of vimentin. On the other hand, by using two different monoclonal antibodies that recognize basic bovine (31) or human (32) cytokeratins we could identify cytokeratin 8 in ASML cells (Fig. 2, E and G, respectively), whereas AS cells did not express detectable levels of this cytokeratin (Fig. 2, F and H). Cytokeratins 17, 18, and 19 were not recognized by these antibodies and the reason for the lack of cross-reactivity with cytokeratin 7 between human and rat is not known. By utilizing antibodies against various intermediate filament proteins we concluded that AS cells express the mesenchymal vimentin type intermediate filaments whereas the ASML variant expresses mainly cytokeratins.

RNA Blot Analysis of Intermediate Filament Protein mRNAs in AS and ASML Cells. We used complementary DNA clones that recognize the basic and the acidic subgroups of cytokeratins in a wide variety of eukaryotic cells (34) for determining the level of mRNAs for cytokeratins in AS and ASML cells. Fig. 3, A and B are RNA slot blots containing serial dilutions of 1:2 of poly(A)-containing RNA from AS and ASML cells, respectively (the upper slot contains 5 \(\mu\)g RNA hybridized with a mixture of \[^{32}P\]nick translated complementary DNAs of both the acidic and basic configuration, the expression of cytoskeletal elements, and the metastatic phenotype of cells.

MATERIALS AND METHODS

Cell Culture. The 10 AS-7 and the 14 ASML-1 cloned cell lines were derived from a spontaneous adenocarcinoma of the pancreas (BSp73) in a BD X rat as previously described (20). The cells were cultured in RPMI 1640 medium (Grand Island Biological Co.) containing 10% heat-inactivated fetal bovine serum (Bio-Lab), nonessential amino acids, and antibiotics. Cells were grown at 37°C in a humidified atmosphere of 5% CO\(_2\)-95% air either on plastic or on plastic coated with 0.1% gelatin (14) for two dimensional isoelectrofocussing and SDS gel electrophoresis. Equal amounts of trichloroacetic acid-insoluble radioactive proteins of total radioactive cell proteins from each cell type were analyzed.

Immunoblot Analysis of Proteins. The proteins of the Triton-high salt insoluble fractions from similar numbers of cells were separated on 10% acrylamide gels and were then transferred electrophoretically to nitrocellulose paper according to Towbin et al. (30). The following antibodies were used in the immunoblot assays: K\(_\alpha\) 18.3, a monoclonal antibody against bovine basic cytokeratins (31); AE-3, a monoclonal antibody against human basic cytokeratins (32); and a monoclonal antibody against vimentin prepared by O. Gigi and B. Geiger (The Weizmann Institute). The second antibody was \(^{125}\)I-labeled goat anti-mouse IgG. Immunoblotting of the Triton-high salt insoluble material enriched in intermediate filaments was performed at 68°C in 5x SSC, 10x Denhardt's solution, and 0.1 SDS for 24-36 h. The filters were washed to a stringency of 0.1x SSC at 53°C.

RNA Extraction and RNA Blot Hybridization. Poly(A)-containing RNA from the cytoplasm of cells was prepared essentially as previously described (15). Briefly, the cells were washed in an ice cold isotonic buffer (150 \(\mu\)m NaCl, 10 \(\mu\)m Tris-HCl, pH 7.5, 1.5 \(\mu\)m MgCl\(_2\)) and then scraped into the same buffer with a rubber policeman. Nonidet P-40 was added to 0.5% and after vigorous mixing the nuclei were sedimented. To the supernatant an equal volume of a mixture containing 7 M urea, 0.35 \(\mu\)m NaCl, 10 \(\mu\)m Tris-HCl, pH 7.5, 1% SDS, and 10 \(\mu\)M EDTA were added to 0.5% and after vigorous mixing the nuclei were sedimented. The Triton-insoluble material enriched in intermediate filaments was solubilized in the sample buffer of Laemmli (28) for SDS polyacrylamide gel electrophoresis or in the lysis buffer of O’Farrell (29) for two dimensional isoelectrofocussing and SDS gel electrophoresis. Equal amounts of trichloroacetic acid-insoluble radioactive proteins of total radioactive cell proteins from each cell type were analyzed.

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Fig. 1. Pattern of intermediate filament proteins in AS and ASML cells. AS (A) and ASML (B) cells were labeled with [35S]methionine and the Triton X-100-insoluble (C and D) and -soluble (E and F) fractions containing similar amounts of radioactivity of AS (C and E) and ASML (D and F) cells were analyzed by two-dimensional gel electrophoresis. a, actin; v, vimentin; t, tubulins; ladder, degradation products of vimentin, cytokeratins 7, 8, and 17-19 according to the nomenclature of Moll et al. (35); ief, isoelectrofocusing.

cytokeratins subfamily. From such analyses we found that there is about 15 times more mRNA coding for cytokeratins in the ASML (Fig. 3B) than in the AS (Fig. 3A) cells. The mRNAs were also resolved on an agarose-formaldehyde gel, transferred to nitrocellulose, and then hybridized with [32P]-labeled complementary DNA to the basic (Fig. 3, C and D) and acidic subfamily of cytokeratins (Fig. 3, E and F). The AS cells (Fig. 3, C and E) showed only background levels of hybridization with both probes, whereas in ASML cells mRNAs specific for both basic (Fig. 3D) and acidic (Fig. 3F) cytokeratins DNA were identified.

In contrast, when a complementary DNA probe specific for vimentin (33) was used, the AS cells (Fig. 3G) showed a significantly stronger signal than did the ASML cells (Fig. 3H). It is therefore concluded that the ASML cells contain mRNAs coding for both acidic and basic cytokeratins, whereas AS cells accumulate only vimentin mRNA.

Modification of Vimentin Synthesis in AS Cells During Suspension Culture. In a different tumor system (B16 melanoma (11), and in a variety of other cell types (12, 13, 16)) we have previously demonstrated that the expression of vimentin and cytokeratins can be experimentally modulated by changing the extent of cell-cell contact or by modulating cell spreading (for review, see Ref. 37). Therefore it was of interest to study the expression of intermediate filament proteins in AS and ASML cells under similar culture conditions. Cells were seeded on plastic plates or on plates coated with poly(HEMA) that prevents cell adhesion, and cell morphology and the intermediate filament protein pattern were determined 3 days later in cells labeled with [35S]methionine. Fig. 4A shows that AS cells form large aggregates on poly(HEMA)-coated plates, whereas ASML cells generally grow as single cells in suspension (Fig. 4B) and occasionally form minute aggregates containing less than 10 cells. It is noteworthy that since both cell types are anchorage independent, they proliferate in suspension as well as in their adherent form. A comparative analysis of the intermediate filament protein pattern of AS cells grown as a monolayer (Fig. 4C) and in suspension on poly(HEMA)-coated plates (Fig. 4D) shows a marked reduction in vimentin synthesis during suspension culture. Vimentin synthesis is already partially restored 4 h after replating the cells on control plastic (Fig. 4E) and is completely restored by 8 h after replating (Fig. 4F). The decrease in vimentin synthesis during suspension culture results from a parallel decrease in vimentin RNA in the cytoplasm of suspended cells (compare Fig. 4, I and J). The recovery of vimentin synthesis after
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In the present study we have demonstrated a differential expression of intermediate filament proteins in two variant cell lines derived from a spontaneous adenocarcinoma (BSp73) of the pancreas from BD X rats. The flat and low metastatic variant (AS) expressed only the vimentin-type mesenchymal inter-

Fig. 2. Immunoblot identification of intermediate filament proteins in AS and ASML cells. The Triton-high salt insoluble fraction from the same number of AS (A, C, F, and H) and ASML (B, D, E, and G) cells was analyzed by SDS-gel electrophoresis. The proteins were identified by Coomassie blue staining (A and B) or by immunoblotting (C-H) with (C and D) monoclonal antivimentin antibody, (E and F) monoclonal anti-bovine basic cytokeratins antibody, and (G and H) monoclonal anti-human basic cytokeratins antibody. h, histones; v, 7, 8, and 17-19, as in Fig. 1.

Fig. 3. The level of intermediate filament proteins mRNA in AS and ASML cells. Poly(A)-containing RNA from AS (A, C, E, and G) and ASML (B, D, F, and H) cells was analyzed by either slot blot hybridization (A and B) with a mixture of *H-labeled complementary DNAs for the acidic and basic cytokeratins as described in "Materials and Methods," or by RNA blot hybridization with a complementary DNA probe for the acidic cytokeratins (C and D) and with a complementary DNA probe for the acidic cytokeratins (E and F). G and H, RNA blot hybridization with a complementary DNA probe for vimentin; kb, kilobase.

replating was followed by a simultaneous increase in vimentin RNA in the cytoplasm (Fig. 4K). In contrast, the spherical ASML cells which have a round configuration both on plastic (Fig. 1B) and in suspension culture (Fig. 4B) display a very similar pattern of cytokeratins on plastic (Fig. 4G) and in suspension on poly(HEMA)-coated plates (Fig. 4H). Thus in ASML cells where cell-cell contact and cell shape could not be altered dramatically by varying the culture conditions, the synthesis of intermediate filaments also remained unchanged, whereas in the AS cells the change from the flat to a spherical configuration in suspension was followed by a dramatic decrease in vimentin synthesis.

DISCUSSION

In several in vitro systems it was demonstrated that the expression of intermediate filament proteins can be modulated. In many epithelial cells, vimentin synthesis began soon after the cells were placed in culture (43). In cells where vimentin is coexpressed with cytokeratins in vivo its expression was demonstrated in epithelial cells that migrate out from the epithelium, as in the case of the parietal endoderm during embryogenesis (44). Similarly in bladder epithelial cells treated with a chemical carcinogen the additional expression of vimentin was observed (45). In established epithelial cell lines in culture that coexpress vimentin and cytokeratins the expression of vimentin was found to be related to the extent of cell spreading or shape (12, 13), whereas the synthesis of cytokeratins was maximal under extensive cell-cell contact (13, 16). Thus a low level of vimentin expression is obtained in cells where culture conditions impose a spherical configuration on cells as demonstrated in B16 melanoma (11) and as we observed in the spherical variant of the BSp73 ASML cell line. In this variant the round phenotype is expressed under all culture conditions. Interestingly, the expression of vimentin could be dramatically reduced in the flat AS

Thus metastatic tumor cells of epithelial origin in ascites or in the pleural fluid were found to express cytokeratins and vimentin, while solid carcinomas expressed only cytokeratins (40). In a study with murine Sarcoma 180, the ascites from this sarcoma were found to coexpress cytokeratins and vimentin, whereas the solid tumor from the same sarcoma expressed only vimentin (41). Furthermore, in rat ascites hepatoma cell lines originating from the same liver carcinoma but with different metastatic capabilities, the spontaneously derived metastatic line was found to coexpress cytokeratins and vimentin, whereas the low metastatic counterpart expressed only vimentin (42). These studies are in line with our findings with the BSp73 adenocarcinoma variants and suggest that differences in the microenvironment from which the cell lines were derived may confer changes in the expression of intermediate filament proteins in the various cell lines.

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variant by placing the cells in suspension (Fig. 4). It is noteworthy that in the BSp73 system the decrease in vimentin synthesis during suspension culture resulted from a parallel decrease in vimentin mRNA in the cytoplasm (Fig. 4), whereas in the B16 melanoma system the decrease in vimentin synthesis during suspension was not followed by a similar decrease in vimentin mRNA translatability in vitro (12). While these differences in the two tumor cell systems in mRNA translatability in vitro and mRNA content as measured by RNA blot hybridization could be real, it is also possible that a difference in the mRNA content in suspended B16 melanoma cells could not be detected by the less sensitive in vitro translation assay (12).

Taken together, it appears that vimentin expression is related to the acquisition of a well spread morphology, and as previously suggested, culture conditions that allow cell attachment and spreading might induce vimentin expression (46). Our attempts to "correct" the defective spreading of ASML cells by seeding them on either bovine endothelial cell-derived extracellular matrix or concanavalin A-coated substrates, or by treating the cells with dibutyryl cyclic AMP were unsuccessful and the cells remained spherical (47). This implies that in ASML cells both the intermediate filament protein pattern and the morphological characteristics are primary manifestations of the cellular phenotype and cannot be modulated by environmental conditions.

Finally, the switches in the synthesis of intermediate filament proteins either by experimental modulation of cell shape and contacts (16) or as displayed by the spontaneous tumor cell variants in this and other studies (48) could be related to the metastatic capacity of the various cell types. The availability of both experimental and spontaneous types of systems might be helpful in further studies aimed to elucidate the relationships among intermediate filament protein expression, cell spreading, and the malignant metastatic properties of cells.

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