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Heterogeneity of Fibroblast Response in Host-Tumor Cell-Cell Interactions in Metastatic Tumors

Mustafa Kh. Dabbous, Lena Haney, Lee M. Carter, A.K. Paul, and James Reger

Departments of Biochemistry (M.K.D., L.M.C, A.K.P.), Periodontics (L.H., M.K.D.), Anatomy (J.R.), and U.T. (M.K.D.), Memphis Cancer Center, The University of Tennessee, Memphis, The Health Science Center, Memphis, Tennessee 38163

The spread and invasion of tumor cells into host tissues are associated with the release of elevated levels of collagenolytic activity of both host and tumor cell origins. However, the mechanisms of regulation of the enzyme activity is still unresolved. Histological examination of human and animal tumors revealed morphological changes in stromal fibroblasts and mast cells at the tumor periphery. Numerous mast cells appeared at microfoci along the tumor: host tissue junction and mast cell degranulation were associated with collagenolysis. In vitro studies, using rat mammary adenocarcinoma and human lung adenocarcinoma cells, showed that both tumor cells and host fibroblasts participate in matrix degradation. Tumor-associated stromal fibroblasts released higher levels of enzyme activity than normal fibroblasts and were more responsive to stimulation by tumor-conditioned media and soluble mast cell products. Host fibroblasts appear to be heterogeneous populations of responsive and nonresponsive subpopulations based on their response to tumor- or mast-cell-mediated stimulation of collagenase release. Fibroblast subpopulations were obtained by density fractionation of serum-deprived, synchronized confluent fibroblasts on discontinuous Percoll gradient. Density-fractionated fibroblast subpopulations differed in their response to stimulation by mast cell products and tumor-cell-conditioned media. The stimulatory activity of tumor-cell-conditioned media also varied as a function of the metastatic potential of the tumor cells. The data suggest that cellular interactions between tumor cells and select subpopulations of host fibroblasts at the tumor periphery play a key role in host tissue degradation. However, heterogeneity of stromal fibroblasts may determine the site and extent of the tissue damage at foci of tumor invasion.

Key words: tumor invasion, cell-cell interaction, fibroblast response, collagenolytic activity, mast cell products

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Invasive neoplasms and metastatic tumor cells may be confronted by a variety of natural tissue barriers in vivo, especially connective tissue stroma and basement membrane structures. As collagen represents the major structural protein of all tissues and provides the chief obstacle to passive migration of tumor cells [1], it has been postulated that collagenolytic enzymes are required to facilitate the spread and invasion of tumor cells into host tissues [for reviews see 2–7]. There is now substantial evidence to support this hypothesis; collagen degradation has been observed at the outer margin of some invasive tumors [7–9], and immunolocalization studies have demonstrated the enzyme collagenase at similar sites [11–14]. The expression of collagen-degrading enzymes is invariably microenvironmental in nature, often occurring at microfoci around the tumor periphery [11], and it seems likely that local host tumor cell interactions are important in modulating collagenolytic activity [15–19]. Work in this laboratory and others showed the enhanced release of collagenolytic activity in cultures of stromal fibroblasts stimulated by tumor cells or their conditioned media [15–17]. Furthermore, mast cell accumulation in increased numbers and degranulation at the tumor periphery in association with stromal lysis have been recently reported [20]. Stromal response at the tumor:host junction, however, appears to be variable [9,20]. Tumor-associated fibroblasts, presumably at the invasion zone, appear to be more responsive to stimulation by tumor cell conditioned media [16] and by mast cell products (MCP), than normal host fibroblasts [20]. It is not known whether the difference in fibroblast response is due to tumor-derived modulation of fibroblast behavior, or to the presence of responsive and nonresponsive fibroblast subpopulations. Because of our interest in the mechanism of tumor invasion and metastasis, especially the cellular interactions involved in matrix degradation, we have focused our attention on stromal fibroblast interactions at the host:tumor interface "invasion zone." In this report, we present data which suggest the heterogeneity of stromal fibroblasts and the presence of subpopulations within apparently homogeneous cell lines which vary in their response to both host- and tumor-derived modulators of collagenolytic activity at the invasion zone. This phenotypic variability may be one of several factors which play a significant role in determining the preferred direction of the invasive tumor growth in vivo.

MATERIALS AND METHODS

Fibroblast Cultures

Normal rat skin fibroblasts (NRS) and rat mammary adenocarcinoma-associated fibroblasts (Ln2-F, Ln3-F) were grown and maintained as described previously [20]. Human fibroblasts from normal lung (LT7, LT9, LT10), embryonal testis (HET1), gingiva (GH8), and basal cell carcinoma-associated fibroblasts (BCE5) were established from explants prepared from surgical specimen provided by the Department of Pathology, University of Tennessee (Memphis, TN). BCE5, HET1, GN32, and GH8 subpopulations were obtained by the Percoll density gradient method and by selective detachment by brief trypsinization. All fibroblasts except rat were grown in Alpha-minimum essential medium (AMEM) supplemented with 10% fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, NY). Rat fibroblasts were cultured in the presence of 10% heat-inactivated fetal calf serum and without antibiotics.

Tumor Cells

Human breast scirrhous (MCF7) and infiltrating ductal (BT20) carcinoma cells were obtained from the EG & G Mason Research Institute (Rockville, MD). Human

lung adenocarcinoma (CaLu6) was kindly provided by Dr. Nathan Sloane, Department of Biochemistry, University of Tennessee (Memphis, TN). MTLn2, MTF7, and MTLn3 clones, derived from the rat mammary adenocarcinoma 13762NF, were provided by Dr. Garth Nicolson, Department of Tumor Biology, University of Texas, M.D. Anderson Hospital and Tumor Institute (Houston, TX). All cells were grown and maintained in the same growth media as described above.

Tumor-Cell-Conditioned Medium

Serum-free conditioned media were collected after 24 hr from tumor cells incubated at 5×10^5 cells per ml of medium. The media were sterilized through a Millex-GS filter unit and stored at $-20°C$. Each conditioned medium was diluted (1:3) with serum-free AMEM before incubating with the fibroblasts.

Mast Cell Products (MCP)

Mast cell products were derived from purified rat peritoneal mast cells collected from rats bearing mammary adenocarcinoma tumors. Enriched mast cell fractions were obtained by Percoll discontinuous density gradient as described previously [21]. The isolated mast cells were adjusted to 4×10^6 cells per ml in 1 M NaCl, extracted overnight at 4°C, and sonicated for 10 sec. The preparation was centrifuged at 16,000 rpm for 1 hr and the resulting supernatant (MCP) was stored at $-20°C$.

Histology

Rat and human tumor specimen with surrounding host tissues were fixed in 1% formaldehyde and 0.25% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C and embedded in JB-4 plastic. Sections (2 μm) were stained according to the method of Bromley et al [22].

Collagenase Activity

For assays, fibroblast cultures were each seeded at 10^5 cells per well in multiwell trays containing growth medium. After 24 hr, the media were removed and replaced with serum-free AMEM with and without a supplement of MCP (10%) or tumor-cell-conditioned medium. Media were collected from triplicate wells at day 3 and stored at $-20°C$. For protein determination, total cell densities were dissolved in 500 μl 0.1 N NaOH per well. Protein concentrations were determined by the method of Lowry et al [23]. Collagenase activity in the serum-free media was assayed by measuring the release of soluble radioactive peptides from 30 μl [^{14}C]-glycine-labeled reconstituted collagen fibril gels after 18 hr at 35°C as described earlier [16]. One unit of collagenase degrades 1 μg of collagen per min at 35°C. Latent collagenase was activated by incubating 20 min with 25 μg trypsin followed by fivefold concentration of soybean trypsin inhibitor.

Fibroblast Subpopulation

BCE5, GH8, and HET1 explants were prepared from the fresh tissue and maintained in AMEM supplemented with 10% FCS. After initial growth, the fibroblasts were harvested and purified using a 2%, 6%, 10%, 14%, 18%, 24%, 30%, 35%, and 40% Percoll discontinuous density gradient. The isolated cells were collected at each interface, washed twice in D-PBS, and plated for growth at 37°C in a

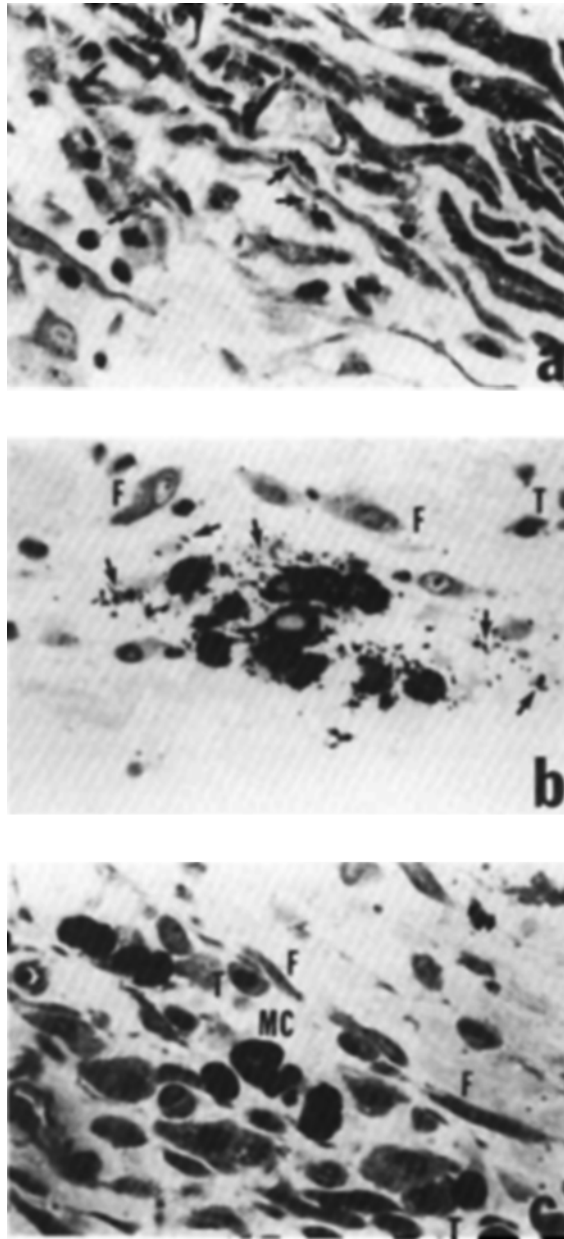

Fig. 1. Photomicrographs of **(a)** stromal connective tissue with mast cells and mast cell granules (arrows); **(b)** a group of mast cells showing degranulation adjacent to enlarged fibroblasts; **(c)** coexistence of intact mast cells (MC), fibroblasts (F), and tumor cells (T). ×360.

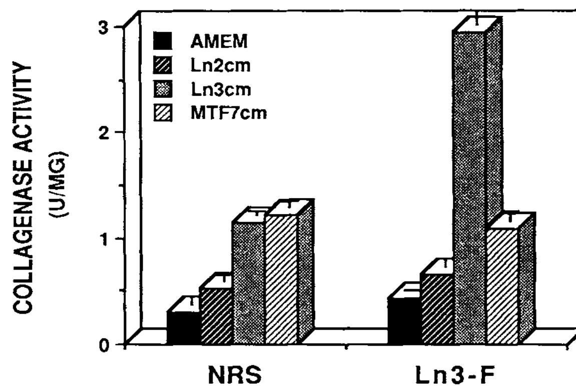

Fig. 2. Effect of tumor-cell-conditioned media on the collagenolytic activity of normal rat fibroblast (NRS), and Ln3 tumor-derived fibroblast (Ln3-F). 24-Hr serum-free conditioned media were derived as described in the text and incubated with fibroblasts for 3 days. Collagenase assay was carried out using 200 μl of medium by the collagen fibril assay using [^{14}C]-labeled collagen.

5% CO_2 humidified atmosphere. Subpopulations were also derived by trypsinization using the method of Hassell and Stanek [24].

RESULTS

Histologic examination of MTLn3 tumor specimens revealed increased number of fibroblasts and mast cells in localized areas of the stroma adjacent to the invading tumor cells. Fibroblasts appeared remarkably large, and mast cell degranulation was often seen associated with foci of matrix degradation along the host:tumor interface (Fig. 1). Similar observations were made in the human tumor specimens (data not shown).

Tumor Cell-Fibroblast Interactions

Normal fibroblasts release very low levels of latent collagenase activity in vitro [16,20]. Tumor cells also release negligible amounts of enzyme activity in the culture media [20]. However, cocultures of the highly metastatic cell variant MTLn3 (Ln3) of rat mammary adenocarcinoma, and normal rat fibroblast (NRS) stimulated the release of relatively higher levels of enzyme activity. The stimulatory effect was not dependent on direct cell-cell contact since media conditioned by the tumor cells (Ln3cm) enhanced the release of fibroblast collagenolytic activity (Fig. 2). The enhancement of in vitro enzyme release was dependent on the nature of the target fibroblast and the metastatic potential of the effector (tumor cell). Thus, stromal fibroblasts derived from the tumor periphery (Ln3-F) were more responsive than normal fibroblasts (NRS), and media conditioned by the highly metastatic clone MTLn3 cells (Ln3cm) were more effective than those of the low metastatic clone variant MTLn2 (Ln2cm). Conditioned media derived from the tumor clone MTF7 (with metastatic potential intermediate between Ln2 and Ln3) appeared to have no differential effect on either normal (NRS) or tumor-derived (Ln3-F) fibroblasts. In this respect, MTF7 and MTLn2 tumor-cell-conditoned media had similar effects. The response of stromal fibroblasts associated with the low metastatic variant MTLn2 (Ln2-F) was not significantly different from that of normal fibroblasts NRS when

stimulated by Ln2 conditioned media (Ln2cm) (Fig. 3). The enhancement of enzyme activity of tumor-associated fibroblasts appeared to be tumor specific and varied with the metastatic potential of the tumor cells.

Fibroblast Heterogeneity

Fibroblast heterogeneity has been examined by density-fractionation of apparently homogeneous population of fibroblasts. Several human fibroblast cell lines were grown to confluence and then serum-deprived to obtain a nongrowing population synchronized in the G1/G0 phase of the cycle [25]. Based on differences in cell density, cells were fractionated into distinct zones at 2–10%, 18%, 24%, and 35% Percoll. These distinct bands were recovered from the gradient centrifugation of several cell lines examined. Differences were observed, however, in the extent and distribution of cell populations among the different zones. The cells removed by aspiration and grown as described in Materials and Methods showed distinct differences in growth rates.

Density-fractionated fibroblast subpopulations differed in size and in morphology (Fig. 4). The fraction separating at 2–10% (B) contained small spindle-shaped cells (major); large, flat, and thin cells (minor); and dendritic cells with long processes (minor). The fraction C, separating at 24%, represents the major population and contains a uniform cell type—mainly short, broad cells with uniform size. The density fraction D, separated at 35% Percoll zone, contained mainly long, spindle-shaped densely packed cells with ruffled edges and long processes. Staining fixed cells with fluorescein-labeled anti-actin stained the cytoskeletal elements in most of the cells in the subpopulations. However, fraction C (24%) was poorly stained with anti-actin antibodies. Staining fixed cells with fluorescein-labeled antimyosin antibody showed no significant labeling of the 24% (C) fraction, while a large number of cells in the 35% (D) fraction were densely labeled, suggesting increased content of myosin-containing structural components in these cells (Fig. 4E,F). The heterogeneity observed in antimyosin staining of cells in this subpopulation may be due to variation in the myofibrillar content or due to microheterogeneity among cells in this subpopulation.

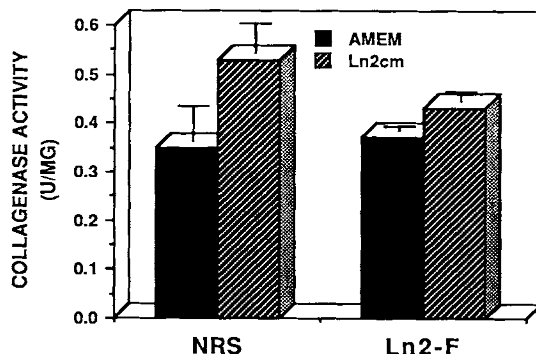

Fig. 3. Effect of MTLn2 tumor-cell-conditioned media on the collagenolytic activity of normal rat fibroblasts (NRS) and Ln2 tumor-derived fibroblasts (Ln2-F). Experimental conditions as in Figure 2.

Heterogeneity of Tumor Cell-Host Fibroblast Interactions

We have previously shown that tumor-associated fibroblasts (TAF) differ in their phenotype from normal fibroblasts [16]. It was suggested that TAF may represent a select subpopulation of responsive fibroblasts or that they may be simply tumor-activated stromal fibroblasts. The response of various fibroblast cell lines and their density-fractionated subpopulations to tumor-cell-conditioned media has been examined. Human fibroblast cell lines HET1, GH8, and BCE and their subpopulations were stimulated by incubation with media conditioned by human lung adenocarcinoma cells (CaLu6) or by basal cell carcinoma cells (BCE). The parental cell line (P) of HET1 did not show any significant effect in the collagenolytic activity after 3 days of incubation with CaLu6-conditioned medium (CaLu6-cm) (showed 5% decrease). However, subpopulations A and C showed significantly high responses, D showed a decrease, while the collagenolytic activity of B, E, and F was nearly unaffected by CaLu6-cm (Fig. 5). The decrease in enzyme activity of subpopulation D in presence of conditioned medium compared to AMEM may be due to a certain degree of inhibition.

The gingival fibroblast cell line GH8 (P), on the other hand, was responsive to stimulation by the lung tumor-cell-conditioned medium (CaLu6) as shown in Figure 5 (bottom l). The relative response of the parental population (P) and its subpopulations when stimulated by CaLu6-cm is also depicted in Figure 5. The pattern of response, in general, differed significantly from that of HET1. While the collageno-

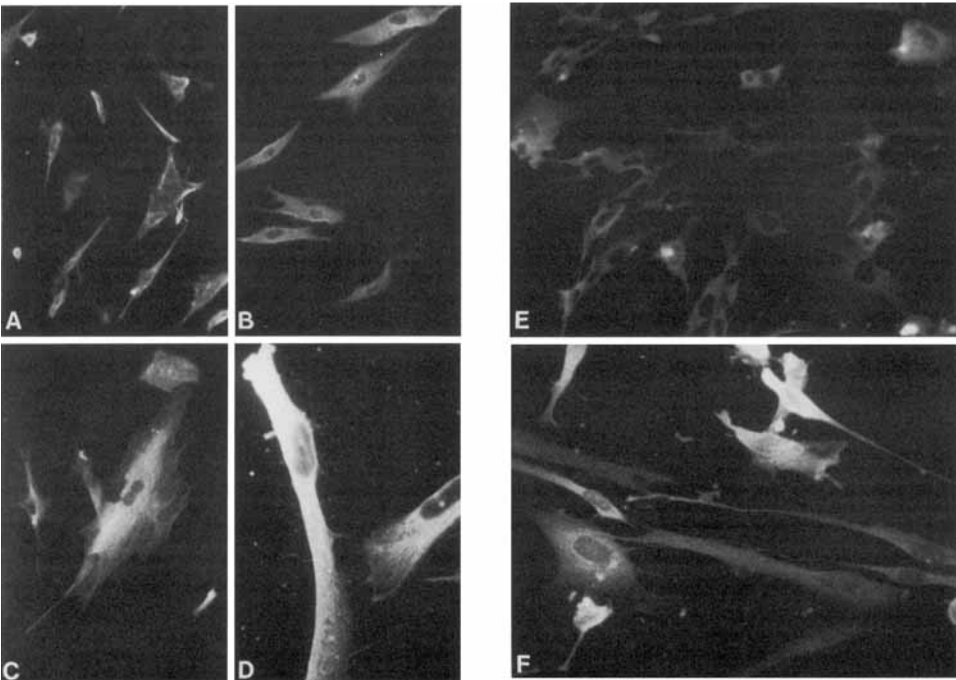

Fig. 4. Fluorescence photomicrographs of human basal cell epithelioma-associated fibroblasts showing in vitro identification of actin and myosin proteins. Indirect immunofluorescence staining with anti-actin: **A:** tumor-associated fibroblast (TAF). Fibroblasts obtained from Percoll gradient zones. **B:** 2–10%. **C:** 24%. **D:** 35%. **E:** 24%. **F:** 35%. (All stained with antimyosin.) A,B,E, ×160. C,D,F, ×400.

lytic activity of subpopulations A, B, and E was enhanced, that of subpopulations C and D slightly decreased while that of F was unchanged (Fig. 5). The data indicated that fibroblast heterogeneity does not appear to be tissue-specific. The presence of certain subpopulations of fibroblasts that are responsive and others that are nonresponsive to tumor-derived soluble factor(s), was also demonstrated among the apparently homogeneous gingival fibroblast cell line GH8 (Fig. 5, bottom).

Tumor-associated fibroblasts (TAF) in the stromal tissue represent the fraction most intimately associated with the tumor cells and appear to play a significant role during invasion and tumor progression. The question of heterogeneity of stromal fibroblasts adjacent to the invading tumor mass was also examined. These fibroblasts appeared to be heterogeneous in their response to tumor-derived soluble factor(s). The enzyme activity of tumor-associated fibroblasts (TAF) derived from basal cell carcinoma was significantly enhanced by BCE-derived conditioned media (Fig. 6). However, only select subpopulations of the density-fractionated tumor-associated fibroblasts were responsive to the BCE tumor-conditioned medium. These responsive subpopulations represented a major portion of the TAF cell population (approximately 60%).

Cellular interactions between tumor cells and host fibroblasts, leading to en-

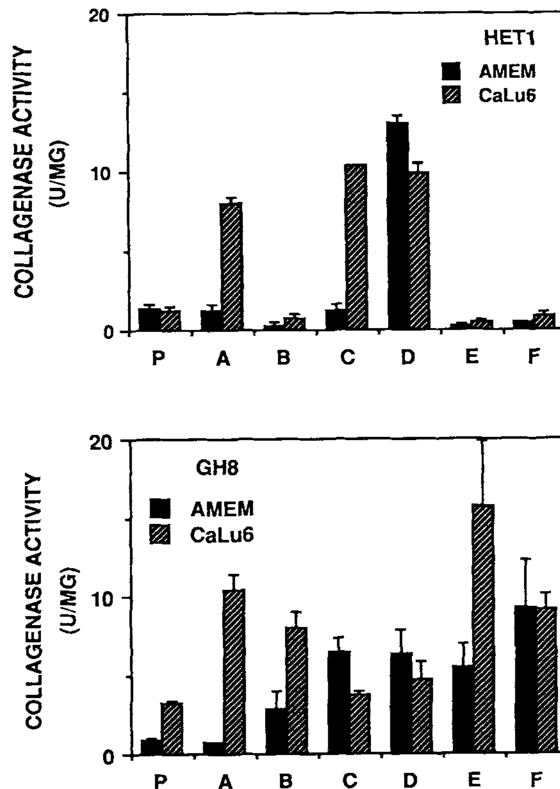

Fig. 5. Effect of human lung adenocarcinoma (CaLu6)-conditioned medium on the collagenase activity of human embryonal testis (HET1) and gingival (GH8) fibroblasts and their subpopulations. Control cultures contained serum-free AMEM. **P** represents the parental cell line and **A–F** represent density-fractionated cell subpopulations separated at 6%, 10%, 14%, 18%, 24%, and 35% Percoll gradient interfaces, respectively. Bars represent the mean values of triplicate assays ± SD.

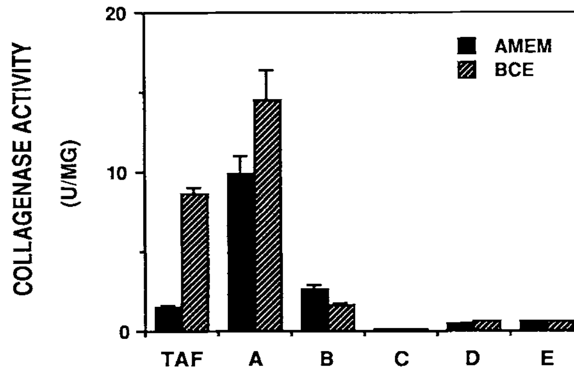

Fig. 6. The effect of basal cell carcinoma (BCE) on tumor-associated stromal fibroblasts (TAF), and density-fractionated subpopulations derived from TAF cells separated at (A) 2–10%; (B) 18%; (C) 24%; (D) 30%; and (E) 35% on Percoll gradient as described in the text. 24-Hr serum-free medium was collected from BCE tumor cell cultures, incubated with BCE-derived fibroblast subpopulations, then media were collected after 3 days and assayed as described in the text.

hanced collagenolysis, may depend to a certain extent on the type of tumor cells from which the conditioned medium was derived and appear to be tumor-specific. Using GH8 fibroblasts and their density-fractionated subpopulations as target cells, it was found that tumor modulation of collagenolytic activity varies with the type of tumor cells (Fig. 7). Less dense fibroblast subpopulations appeared to be more responsive to lung adenocarcinoma CaLu6-cm than to breast carcinoma. High-density subpopulations, except for subpopulation E, appeared to be more responsive to both tumor cell types. Fibroblast subpopulations appear to be selective in their response to tumor-derived soluble factor(s).

DISCUSSION

The spread and invasion of tumor cells into host tissues appear to be facilitated by the release of elevated levels of collagenolytic enzymes [2–6]. The enhanced release of these enzymes appears to be responsible for most of the stromal tissue damage frequently observed at the tumor periphery [1–9,26]. The enzyme production is microenvironmental in nature [11], often occurring at microfoci along the tumor periphery, and it is likely that host-tumor cell interactions play a major role in modulating the collagenolytic activity [15–17,28–32]. It has been previously demonstrated that tumor cells or their conditioned media stimulated the collagenolytic activity in cultures of host fibroblasts [15–17]. Epithelial-mesenchymal interactions have been described earlier [29]. Although Gross et al suggested that tumor products may mediate the interaction between tumor cells and host cells [30], a mechanism for in vivo modulation of collagenase synthesis is as yet not known. It may be postulated to occur through elaboration of a factor, possibly by the tumor acting directly to increase synthesis of collagenolytic enzymes or through a decrease in suppressor mechanisms for active enzyme synthesis. Stimulation of collagenase release by basal cell carcinoma-derived fibroblasts [15], rabbit synovial fibroblasts in response to both animal and human tumor cells [17], and by VX-2-derived fibroblast-like cells (F-cells) stimulated by homologous tumor-conditioned medium [16], demonstrated the importance of tumor cell-host fibroblast interactions in the collagenolysis of host tissues.

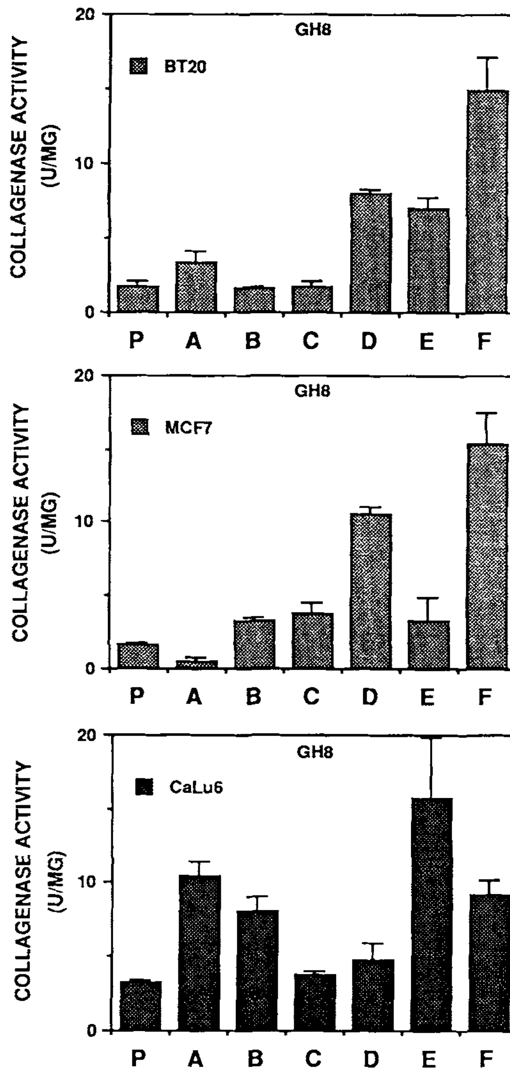

Fig. 7. Response of human fibroblast (GH8) and subpopulations to human breast (BT20, MCF7) and lung (CaLu6) carcinomas. Fibroblast subpopulations **A–F** were produced and maintained as described in the text. 24-Hr serum-free media were collected and incubated with the target cells in multiwell trays for 3 days, as described in the text.

Using rabbit VX-2 carcinoma and associated rabbit fibroblasts, we have reported that both tumor cells and host fibroblasts were capable of releasing collagenase activity in vitro [16]. Data presented in this report showed that, although the level of collagenolytic activity produced by in vitro cultures of rat mammary adenocarcinomas was relatively low, media conditioned by these cells had the potential to stimulate the enzyme activity of syngeneic host fibroblasts. The stimulation was dependent on the metastatic potential of the tumor cells, thus Ln3cm was more effective than Ln2cm. Although MTF7 had metastatic potential intermediate between that of MTLn3 and MTLn2, its ability to stimulate fibroblast collagenase activity was higher than pre-

dicted in comparison with Ln3cm and Ln2cm. However, MTF7 is a clone derived from the parental cell population at the primary site and differs from the lung metastasis variants Ln3 and Ln2 [31]. Media conditioned by the low metastatic variant MTLn2 or by MTF7 cells appeared to have no differential effect on either NRS or Ln3F. Furthermore, MTLn2-associated fibroblasts Ln2-F did not differ significantly from NRS in their response to tumor-derived conditioned media. These observations suggest that tumor cell-host fibroblast interactions appear to be tumor specific. The stimulatory activity depends also on the nature of the target fibroblasts, as histologic observations suggested the unique morphology of fibroblasts at the tumor periphery. Biochemical data showed that MTLn3 tumor-associated fibroblasts (Ln3-F) were more responsive to stimulation by Ln3cm than normal syngeneic fibroblasts (NRS).

Host tissue degradation appears to be localized at certain foci along the tumor periphery. Selectivity in interactions at the site of tumor invasion or preferred collagenolysis site may indicate specificity in tumor cell-host cell interactions. Such interactions may depend on the distribution of cell populations at the invasion zone [18]. Tumor-associated stromal fibroblasts may represent activated fibroblasts or a select subpopulation of responder cells at the invasion zone. The data indicated that certain stromal fibroblasts appear to be activated by adjacent tumor cells or their products, and hence are more responsive to tumor-mediated stimulation than normal fibroblasts. Alternatively, host fibroblasts are heterogeneous and tumor cells or their products may change the microenvironment in their vicinity to become more selective for a given subpopulation of fibroblasts which is more responsive to tumor-derived stimulation. The concept of responder and nonresponder fibroblast subpopulations in an apparently homogeneous mass culture is well supported in the literature [32–35]. Phenotypic variability in fibroblast cultures has been recently described, and density fractionated fibroblasts were shown to consist of distinct cell fractions [36] which were morphologically and biochemically different. Our biochemical and morphological data showed phenotypic variability among density-fractionated subpopulations of stromal fibroblasts. The fibroblast subpopulations showed selectivity and varied in their response to tumor-derived soluble factors (conditioned media). Preliminary binding studies with a partially characterized, tumor-derived cytokine, suggested that tumor-derived subpopulations of fibroblasts showed different specific binding properties towards the cytokine (unpublished data).

The role of fibroblasts in tumor invasion and progression does not appear to be a passive one. It has been recently reported that stromal fibroblasts facilitate tumor take and engraftment [27] and shorten the lag period for tumor growth and increase the growth rate. These authors showed that the addition of fibroblasts to tumor cells was necessary for tumor growth when the number of tumor cells alone was insufficient to produce tumors. Interactions between tumor cells and fibroblasts may be necessary in order for tumor-derived cells to initiate a tumor graft in the host. Certain subpopulations of stromal fibroblasts may play a significant role in tumor invasion, in response to tumor-mediated changes in the microenvironment. Fibroblast heterogeneity and selectivity in the response of morphologically and functionally different subpopulations appear to play a key role in local host-tumor interactions modulating collagenolysis at sites of matrix degradation in the invasion zone.

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