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ARTICLE

Fibroblast Enhancement of Tumor Invasion in a Tumor-Host Interface Recapitulated *in-vitro*

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Tumor cells and fibroblasts were isolated from the tumor-host interface of a colon 4047 tumor growing subcutaneously in a Fischer 344 rat. The populations were co-cultured to recapitulate the tumor-host interface *in vitro*. The co-cultured populations grew in a predictable pattern with tumor cells forming nodules surrounded by fibroblasts. Population dynamic experiments demonstrated the fibroblasts enhanced the growth of the tumor cells but tumor inhibited and ultimately destroyed the fibroblasts. Video microscopic examination of the fibroblasts demonstrated intense membrane ruffling adjacent to the tumor nodules followed by membrane fragmentation and detachment. Immunohistochemical staining for gelatinase A was markedly positive within the fibroblasts surrounding the tumor nodules; but negative within the tumor and in fibroblasts when tumor was absent. This technique recapitulates many aspects of the tumor-host interface *in vitro* and may be a useful model for evaluating several aspects of tumor-host interaction. (Pathology Oncology Research Vol 2, No3, 151–156, 1996)

Key words: invasion, cartilage, pannus, matrix metalloproteinases, cytokines

Introduction

Malignant tumor development requires the neoplastic cell population to escape the usual cellular constraints and invade and destroy adjacent normal tissue. Mathematical models of the tumor host interface[1] demonstrate the importance of peritumoral normal tissue as a potential barrier to tumor invasion. Critical factors necessary for invasive behavior include persistent stimulation of tumor proliferation and breakdown of extracellular matrix within the normal tissue allowing tumor penetration of the "barrier".

The importance of the fibroblast-tumor interaction in this barrier is emphasized by the frequent observation of a brisk fibroblast response with varying degrees of encapsulation in pathologic examination of clinical tumors. Thus, the tumor-host interface often consists of tumor cells in direct contact with fibroblasts, suggesting the interactions of these cells may be a critical factor in determining the effectiveness of the tissue resistance to tumor invasion.

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Although tumorigenesis is ultimately controlled by the biological properties of tumor cells, it appears that the role of host mesenchyma is not entirely passive and not necessarily resistant to tumor growth.[2-4] Several studies of tumor-fibroblast interaction[2-4] have demonstrated increased tumor growth due to the presence of fibroblasts or fibroblast products. The mechanism appears to be the production of stimulating factors by fibroblasts which serve as a persistent stimulus to tumor growth. Most of these studies, however, have used tumor and fibroblast cell lines which may limit their application *in vivo*. Furthermore, virtually no work has been performed on the effects of tumor or tumor products on fibroblast growth.

In addition, a characteristic of an invasive tumor is the production of a variety of factors which break down the extracellular matrix. The positive and negative regulation of these factors appears critical in tumor growth and metastasis.[5-7] Several studies[8-15] have focused on tumor production of matrix metalloproteinases which seem required for invasion and which can be reversed with metalloproteinase inhibitors[16-18] Particular interest has focused on gelatinase A which catalyzes the breakdown of collagen IV,[8] a major component of the extracellular matrix at the tumor-host interface. This suggests that tumor-associated

fibroblasts may serve the host defense both by laying down a barrier of collagen and by producing metalloproteinase inhibitors.[17] Ironically, however, although gelatinase A may be produced by tumor cells, several *in vivo* studies have demonstrated that the major sources of the enzyme in and around tumors are host cells, particularly fibroblasts.[8-10] The mechanism by which tumors promote gelatinase A production by normal fibroblasts is not yet clear. Possible promoters include tumor generated polypeptides or other soluble factors,[11] tumor-induced interstitial acidosis,[14] mechanical stress on host cells caused by crowding or increased interstitial pressure.[15]

We have attempted to develop an *in vitro* model of these aspects of the tumor-host interaction by recapitulating the tumor-fibroblast interface using tumor cells and fibroblasts cultured from an invasive *in vivo* tumor.

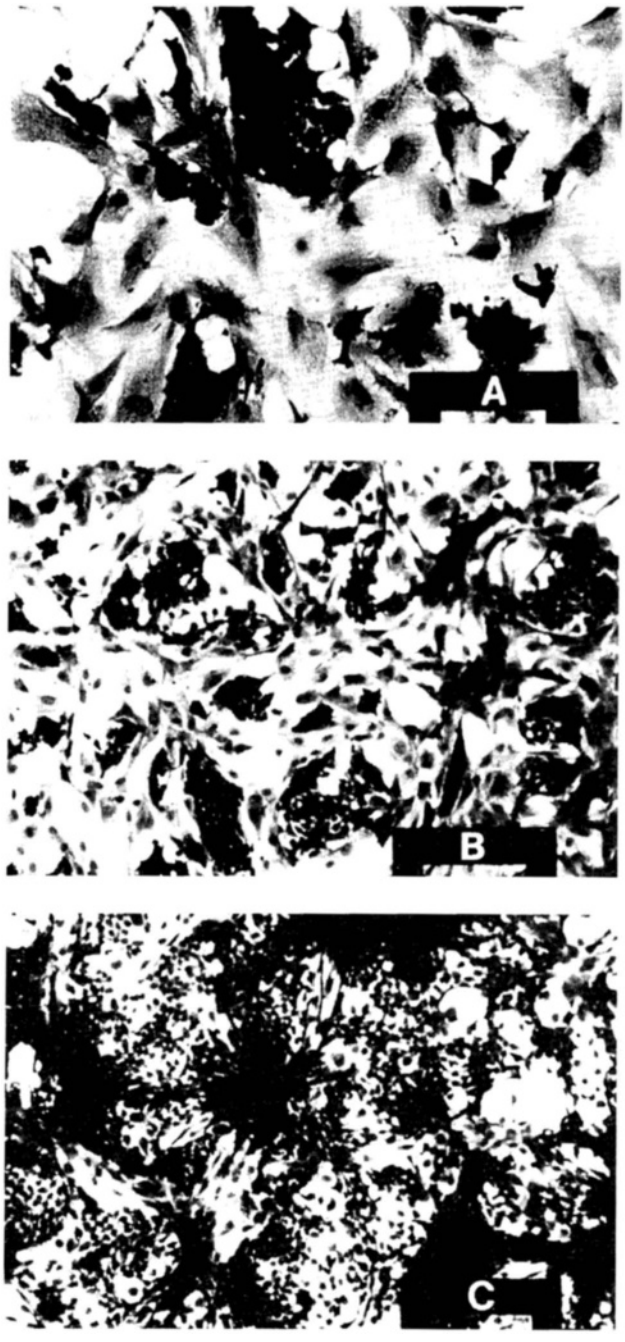

Figure 1. *Photomicrographs of co-cultures of rat colon carcinoma and fibroblasts stained with H & E, after 48 hr (A); 72 hr (B); and 96 hr (C). Subsequent to the initial seeding at a 1:1 ratio, the darkly stained colon carcinoma cells form clusters, which are completely surrounded by fibroblasts. Note the decline in fibroblast population and large coalescent tumor nodules at 96 hours. Microscope measure bars = 100 μm in A; 200 μm in B and C.*

Materials and Methods

Tumor and normal cell cultures

This study utilized the 1,2-dimethylhydrazine induced rat colon adenocarcinoma 4047. The tumor was serially passed *in vivo*. 1- to 3 mm^3 pieces of tumor implanted in the subcutaneous tissues (via trocar) in the flank of male Fischer 344 rats (300 g) resulted in tumor growth in 100% of the animals. The tumors grew to 4 to 5 cm in diameter within 6 weeks. Microscopic examination of the tumor-host interface demonstrated a fibroblastic response forming a thin peritumoral capsule which is penetrated by nests of tumor cells.

To establish primary cultures of the 4047 tumor for *in vitro* studies, 1 mm^3 sections of viable tumor were removed and mechanically disrupted under sterile conditions. These cells were then maintained in DMEM supplemented with 10%fetal bovine serum, 5 μg/500 ml EGF (Sigma Chem Co., St. Louis, MO), 8 mM glutamine, 1mM pyruvate, insulin-transferrin-selenite (ITS, Sigma) 5 ml/500 ml supplement, 50 μg/ml streptomycin, 100 μg/ml kanamycin, and 50 IU/ml penicillin and incubated at 37°C in a humidified 5% CO_2 atmosphere and passed serially. Periodically, 10^6 cultured cells were reinjected subcutaneously into Fischer 344 rats which formed tumors identical to those maintained *in vivo* by serial passages.

Normal rat fibroblasts were also established as primary cultures from subcutaneous connective tissue at the tumor-host interface in tumor-bearing male Fischer 344 rats. For all experiments, the fibroblasts were maintained in DMEM media with 10% fetal bovine serum.

Co-culture experiments

An equal number of colon 4047 cells and fibroblasts ($2x10^5$ cells/line) were seeded together in each well of a 6 well dish in DMEM with 10% FBS and 0.1% Gentamycin and incubated at 37°C in a humidified 5% CO_2 atmosphere. As controls 2 x 10^5 colon 4047 cells and 2 x 10^5 fibroblasts were seeded separately under identical

conditions. A coverslip was placed in the bottom of the dish prior to addition of the cells. To form an extracellular matrix on the coverslips, each was covered with a thin layer of FBS which was then allowed to air dry under sterile conditions. Coverslips were removed at 24 hour intervals following seeding and fixed in 3.7% formaldehyde/PBS for 5 minutes. Some coverslips were stained with hematoxylin and eosin and others underwent immunofluorescence staining as described below.

In some experiments the interface of the tumor nodule and fibroblast capsule was continuously observed and video recorded over a 24 hour period using a phase contrast microscope placed in the incubator. Daily observations of the same tumor nodules were also performed by light microscopy with Nomarski optics.

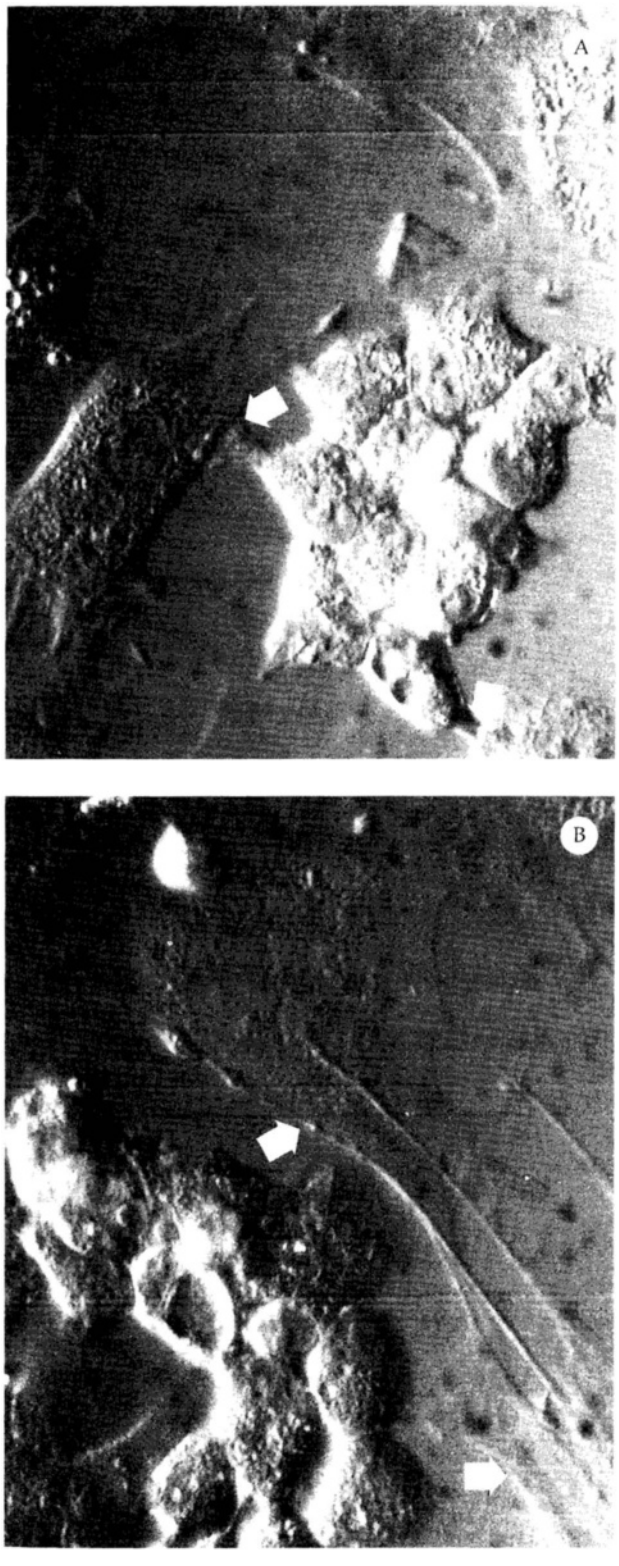

Figure 2. Light microscopic view, imaged with Nomarski optics, of rat colon carcinoma and fibroblast co-cultures (A,B). Panel A shows, after 24 hr, the direct interaction and contacts between well spread fibroblasts surrounding islands of colon carcinoma cell (white curved arrows). In panel B, after 60 hr, direct contacts between the two cell types are rarely observed. Ruffled, retracting membranes are seen in the fibroblasts surrounding the tumor cells, and a distinct distance appears between the two cell types (indicated by the black curved arrows).

Immunofluorescence microscopy

Indirect immunofluorescence microscopy was performed on all three cell co-culture conditions, while grown on glass coverslips to approximately 70% confluence. For the detection of vimentin, the V9 mouse monoclonal antibody (DAKO Corp., Santa Barbara, Calif.) was diluted 1:50 in CMF-PBS. Indirect immunofluorescence staining of keratin intermediate filaments used the mouse anti-human keratin 18 CK5 antibody (ICN). The rabbit polyclonal Ab for type IV collagenase/Gelatinase A was kindly provided by Dr. Stetler-Stevenson (National Cancer Institute, Bethesda, Maryland). This affinity purified antibody has been previously described,[19] and was applied to coverslips subsequent to fixation with 3.7% formaldehyde/PBS.

All coverslips were rinsed in CMF-PBS three times following incubation with the appropriate primary antibodies, and then treated with the appropriate rhodamine or fluorescein-conjugated secondary antibody. For controls, either the primary experimental antibody was omitted, and the cells were incubated with the secondary antibody alone, or a nonspecific mouse or rabbit IgG antibody was used, followed by the appropriate secondary antibody. The cells were viewed and photographs were taken on a Zeiss standard 18 fluorescence microscope with Ilford ASA 400 black and white film.

Cell counting

The number of cells on each coverslip was estimated by counting the cells in 20 high power fields, randomly chosen throughout the coverslip. In co-culture ex-

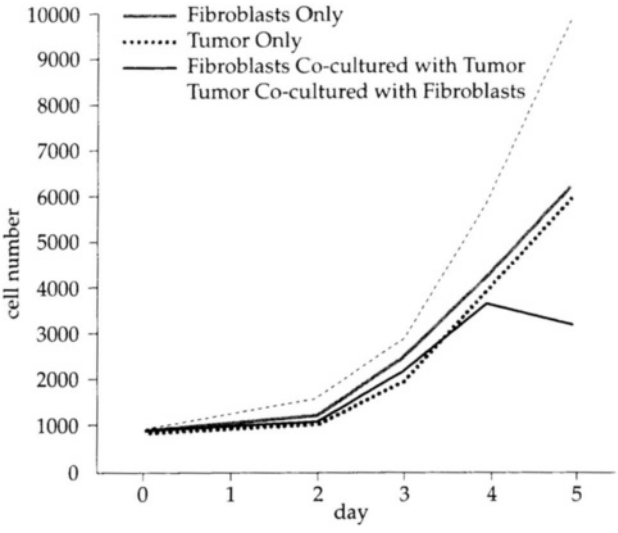

Figure 3. The population dynamics of co-cultures of fibroblasts and colon 4047 cells are compared to each population grown separately. In early, uncrowded conditions the populations have no observable effect on each other. However, as cell density increases, the growth rate of the tumor becomes greater than that seen in the absence of fibroblasts. The fibroblast population slows its growth rate and then steeply declines.

periments, cellular morphology allowed fibroblasts to be differentiated from 4047 colon cells so that each population could be counted separately. Morphologic criteria were subsequently confirmed with vimentin and keratin staining.

Results

Cell growth

The cultures of tumor cells alone and fibroblasts alone demonstrated rapid growth forming typical monolayers which progressively overgrew the coverslip. In the co-culture experiments *(Fig.1)*, the cells were seeded in single cell suspension and were initially distributed randomly throughout the dish in a pattern similar to that seen with the individual cultures. However, 24 hours following seeding, an organizational structure became apparent with tumor cells forming multiple coalescent nodules consisting (usually) of 4 to 10 cells surrounded by scattered fibroblasts.

At 48 hours, the average tumor nodule had expanded significantly but was surrounded by a ring of densely packed fibroblasts forming a well defined capsule. By 96 hours, the tumor nodules had further expanded, and there was a significant loss of fibroblasts from the "capsule". Video microscopy of the fibroblasts at the tumor-host interface during this time period demonstrated intense membrane ruffling with fragmentation of the fibroblast membrane leading to retraction of the fibroblasts away from the tumor cell edge *(Fig.2)*. Ultimately, the fibroblasts were observed to detach from the coverslip and float freely in the media.

Quantitation of the cultures *(Fig.3)* demonstrate that fibroblasts and tumor cells, seeded alone, grew at similar rates. Co-cultured cells initially doubled at rates identical to those seen when the cells were allowed to grow separately. However, after 3 days, co-cultured tumor cells began to grow faster than those cultured alone, while the numbers of fibroblasts leveled and then declined.

Fluorescence staining

As demonstrated in *Fig.4*, keratin staining, indicative of epithelial cells, was observed only in the tumor nodules. No isolated tumor cells were observed in the surrounding fibroblasts. Similarly, vimentin staining, characteristic of mesenchymal cells, was not observed in the tumor nodules and seen only in the surrounding fibroblasts. Thus, the complete segregation of the two cell types was confirmed.

Intense staining for gelatinase A was demonstrated within the fibroblasts surrounding the tumor nodules *(Fig.4)*. Only background to little staining for gelatinase A was observed in the tumor nodules within the co-culture experiments or when the tumor cells or fibroblasts were grown separately.

Discussion

Invasive, malignant growth requires tumor cells to penetrate the potential barrier formed by the surrounding normal tissue. Critical components of this invasion include the degradation of the extracellular matrix, particularly the basement membrane, and the interaction of the transformed cells with normal cells (particularly fibroblasts) at the tumor-host interface.

The matrix metalloproteinases, a family of enzymes which degrade proteins found in the extracellular matrix, have been significantly associated with tumor progression and invasion. In particular, synthesis of gelatinase A (72kDa type IV collagenase) has been shown to correlate with invasive and metastatic behavior *in vitro* and *in vivo*.[8-10] Gelatinase A may be produced directly by tumor cells *in vitro*. However, several studies in human cancer, including colon, breast, head and neck, and skin have shown that the *in situ* sources of gelatinase A are normal host cells, particularly stromal fibroblasts.[8-10] The mechanism by which tumor cells stimulate fibroblasts to secrete collagenase IV remains unclear.

Co-culture experiments have been used to explore the relationship between tumor cells and normal fibroblasts. These have shown that the presence of fibroblasts enhances the growth of tumors probably through the production of soluble factors which have not yet been characterized.[2-4] These studies may be limited, however, because they use culture

cell lines and thus may not be relevant to *in vivo* interactions. Furthermore, the effect of tumors on normal fibroblasts in these co-culture experiments has not been established.

In this study, we were able to create a model to recapitulate the tumor-host interface *in vitro* by establishing in culture the cells which actually form the interface *in situ*. Furthermore, we were able to mimic invasive tumor behavior *in vitro* and show that under these conditions successful tumor "invasion" is at least in part related to the production of gelatinase A. Moreover, in this model, the source of gelatinase A at the tumor-host interface is normal stroma, although the presence of tumor cells is required to induce gelatinase A production. This model also demonstrates enhanced tumor growth in the presence of normal fibroblasts and perhaps more importantly, the destruction of host fibroblasts mediated by the tumor cells.

We believe that this experimental model will be useful in further *in vitro* studies to better define mechanisms underlying tumor invasion. Specifically, it appears to have significant potential with respect to the investigation of

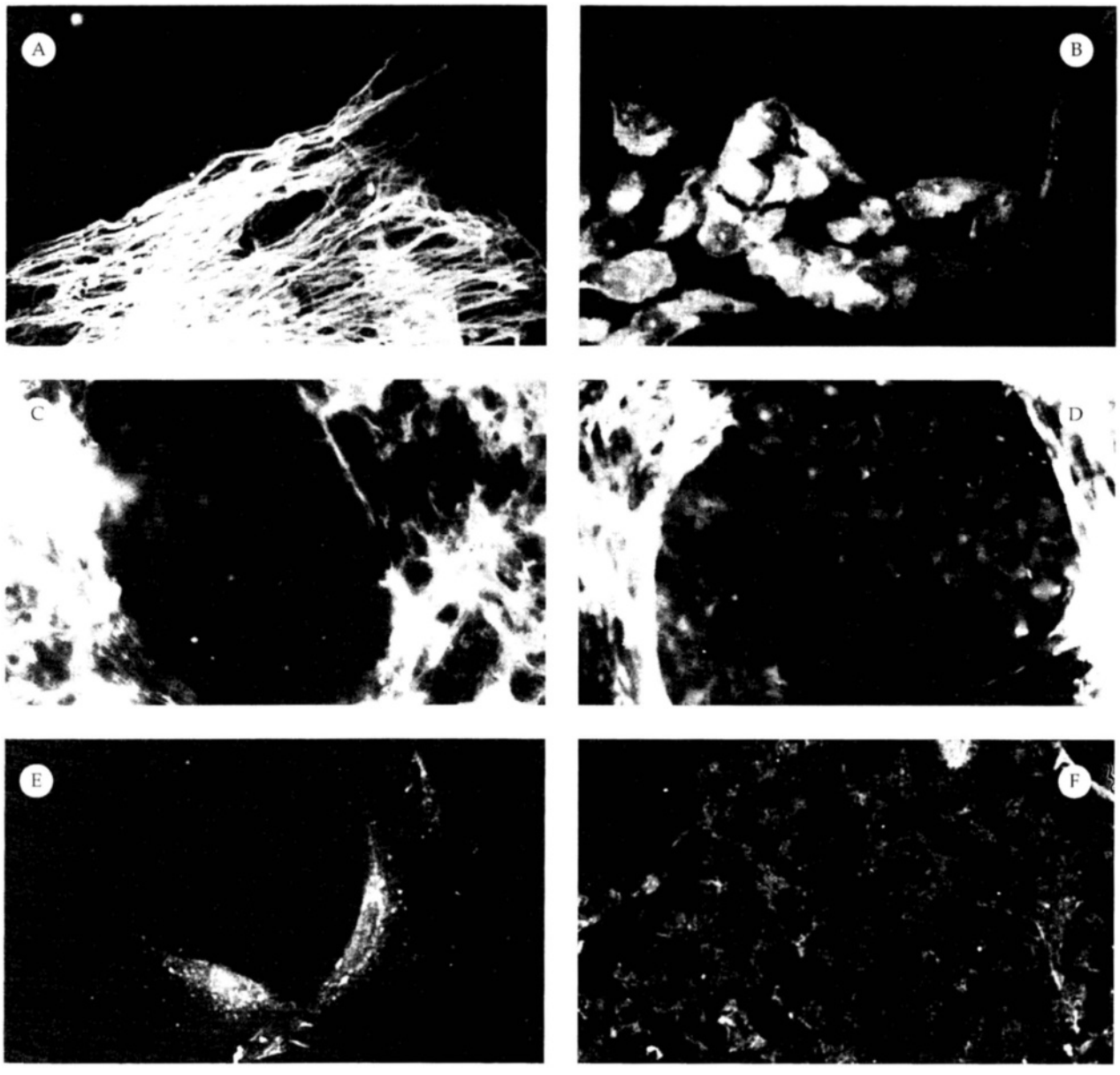

Figure 4. *Indirect immunofluorescence microscopy localization in co-cultures of rat colon carcinoma and fibroblasts (A-D), fibroblasts alone (E), and colon carcinoma alone (F). Panel A depicts Vimentin staining over the fibroblasts; B shows keratin localization restricted to colon carcinoma cells; C shows fibronectin staining over the fibroblasts only; D depicts Gelatinase A over the fibroblasts; E demonstrates minimal Gelatinase A staining in the fibroblasts grown independently; and F shows background levels of Gelatinase A in the colon carcinoma cells cultured alone (x800).*

several critical factors in tumor-host interaction including: 1. the role of metalloproteinases in the facilitation of tumor invasion; 2. the mechanism by which tumor cells induce metalloproteinase production in normal stroma; 3. the role of normal fibroblasts in the growth of tumor cells; and 4. the mechanisms of tumor cell mediated destruction of surrounding host cells.

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