Invasion of an Artificial Blood Vessel Wall by Human Fibrosarcoma Cells

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ABSTRACT

Artificial blood vessel walls constructed by the addition of bovine arterial endothelial cells to multilayers of rat smooth muscle cells were used as substrates for the human fibrosarcoma cell line HT1080. The extracellular matrix proteins elaborated by the smooth muscle cells were prelabeled with [3H]-proline; therefore, their subsequent digestion could be followed by the appearance of radioactivity in the culture medium. The fibrosarcoma cells rapidly hydrolyzed smooth muscle multilayers in the absence of endothelial cells, but an endothelial layer markedly retarded the destructive ability of the tumor cells. The protective effect of the endothelium was not due to a lack of penetration of this cell layer, since HT1080 cells were observed by light and electron microscopy to be in the subendothelial area 24 hr after plating. Subsequently, the tumor cells multiplied in the region between the endothelial and smooth muscle layers and, although their degradative ability was retarded, they were ultimately capable of destroying the structure. Endothelial cells also inhibited hydrolysis of the smooth muscle layers if added simultaneously or up to 1 week after HT1080 cells, but the degree of inhibition was not as great as that seen with a preestablished endothelial layer. Measurable inhibition of tumor cell degradative activity was observed at fibrosarcoma:endothelial cell ratios of 25:1, demonstrating the potency of endothelial cells in modulating this aspect of the invasive phenotype. Although the HT1080 cells only slowly degraded the preexisting matrix proteins in artificial vessel wall cultures, they interfered with the production of new connective tissue proteins which occurred in control cultures. These experiments therefore suggest that endothelial cells have profound effects on tumor cell proteolytic activity, and the significance of these observations to tumor cell extravasation in vivo is discussed.

INTRODUCTION

The exit of malignant cells from the circulation is an important requirement for the successful establishment of a secondary tumor during blood-borne metastasis. Most of our knowledge of extravasation comes from morphological observations of metastasis in animal tumor models (4), and little is known of the cellular and biochemical properties of tumor cells which are responsible for this process. This has been due primarily to the difficulty in obtaining suitable model systems that allow for simultaneous morphological and biochemical observations.

The process of extravasation in vivo begins after the adherence of circulating tumor cells to vascular endothelium, and there seem to be several routes by which malignant cells cross the endothelium and gain access to extravascular tissues. Several studies have indicated that the endothelium is breached between cell-cell junctions (7, 16, 21), although it is not clear whether the tumor cells induce endothelial retraction or take advantage of naturally occurring gaps (22, 25). Other studies have suggested that metastatic cells might follow the path set by emigrating leukocytes or lymphocytes (19, 26), migrate directly through endothelial cell cytoplasms (3) or cause the cytotoxic or enzymatic lysis of endothelial cells (5, 23). Once tumor cells have penetrated the endothelium, they often become sealed off from the circulation by endothelial cells (16, 24, 26) and begin to form secondary tumors.

Recently, several investigators have studied the interactions of invasive cells with endothelial cell layers (1, 13, 14, 27). These studies have been promising in that some of the morphological features of extravasation in vivo appear to be mimicked in vitro. The adherence of tumor cells to the subendothelial matrix produced by cultured cells (13) and hydrolysis of this matrix have also been investigated (10, 15) in order to understand more fully extravasation in the animal. This report extends these studies to include the interaction of a human fibrosarcoma cell line [HT1080 (17)] with an artificial blood vessel wall.

The artificial blood vessel wall was constructed by the growth of bovine arterial endothelial cells on a preformed layer of rat smooth muscle cells to form a structure with several of the morphological and biochemical properties of a vessel wall (8). These structures were then used as substrates for the human fibrosarcoma cell line which was previously found to be capable of the complete degradation of both endothelial (10) and smooth muscle cell (9) extracellular matrices. The use of this model system allowed us to integrate morphological and biochemical events and demonstrated that normal cells, such as endothelial cells, can modulate tumor cell invasive and degradative ability.

MATERIALS AND METHODS

Production of Substrates. Rat smooth muscle cells of the R22CID strain (12) were grown in Eagle’s minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal calf serum ( Irvine Scientific, Irvine, Calif., 2% tryptose phosphate broth (Difco Laboratories, Inc., Detroit, Mich.), penicillin (100 units/ml), and streptomycin (100 μg/ml). Stock cultures were maintained in 75-cm² plastic flasks, and cells derived from them were seeded into 35-mm dishes (2 × 10⁷/dish) for the production of tumor cell substrates. The cultures received ascorbic acid daily (25 μg/ml) and were labeled with L-[3,4-3H]proline (1 μCi/ml; New England Nuclear, Boston, Mass.). Culture medium was changed twice a week, and the addition of [3H]-proline was discontinued 6 days after seeding. Medium was changed 3 times over the next 6 days to remove as much soluble radioactivity as possible, and the labeled smooth muscle multilayers were used as substrates for other cell types as described in the text. Artificial vessel walls were constructed by the addition of 10⁷ bovine arterial endothelial cells of the A14CL-1 strain to these preestablished multilayers as

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RESULTS

The release by HT1080 cells of radioactivity from cultures of smooth muscle cells prelabeled with [3H]proline is shown in Chart 1. The rate of degradation was dependent on the number of fibrosarcoma cells added, and cultures seeded with \(2 \times 10^4\) or \(10^6\) tumor cells were completely destroyed after 18 days of incubation. The destruction of the smooth muscle cell multilayer could be observed directly with phase-contrast optics, and holes in the multilayer appeared approximately 11 days after the addition of tumor cells. Perforations in multilayers seeded with \(4 \times 10^4\) and \(8 \times 10^5\) HT1080 cells, on the other hand, did not appear until later in the experiment. The release of radioactivity from the smooth muscle cell multilayers reflected the hydrolysis of the constituent connective tissue proteins since enzymatic analysis of the residual matrix proteins obtained by NH\(_4\)OH hydrolysis at the end of the experiment showed that 95% of the glycoproteins, elastin, and collagen had been solubilized by the fibrosarcoma cells (data not shown, but see also Fig. 1).

Smooth muscle multilayers containing preformed endothelial layers (artificial vessel walls) were almost completely refractory to hydrolysis by fibrosarcoma cells (Chart 2). The protective effect of the endothelial layer was directly visualized when the cultures were lysed by NH\(_4\)OH treatment at various times after adding HT1080 cells in order that the residual-insoluble proteins could be stained with Coomassie blue (Fig. 1). Cultures containing only smooth muscle cells showed increases in the amounts of insoluble matrix proteins during the 22 weeks of this experiment. The smooth muscle cells and extracellular matrix were completely destroyed by the added fibrosarcoma cells; therefore, virtually no insoluble proteins remained associated with dishes lysed 4 weeks after the beginning of the experiment. Artificial vessel walls contained more insoluble matrix proteins than did smooth muscle cells only, and diges-
tion of these structures by HT1080 cells was retarded markedly. Holes appeared only after 17 weeks of incubation (versus 4 weeks for smooth muscle cells), but considerable destruction of the artificial vessel wall had occurred after 22 weeks. Viable HT1080 cells were isolated from such cultures on several occasions even after these prolonged incubation times.

The interactions of HT1080 cells with these 2 substrates were also investigated by light and electron microscopy after fixation of the cultures in situ at various times after the addition of tumor cells. HT1080 cells attached to and occasionally spread out on the surface of the smooth muscle cells within 24 hr but failed to invade the underlying tissue (Fig. 2a). Electron microscopy showed the tumor cells spread on top of the smooth muscle cells and also their typical morphology including many plasma membrane projections, but no defined attachment sites between the 2 cell types were observed (Fig. 3a).

In contrast to this, HT1080 cells had penetrated the layer of endothelial cells of an artificial blood vessel wall and invaded the subendothelial space within 24 hr (Fig. 2b), although they were seen also on top of the structure. When viewed in the electron microscope, the tumor cells had infiltrated between the endothelial cells which then often resulted in a noncontinuous endothelium (Fig. 3b). The tumor cells adopted a more flattened appearance with many membrane projections and were coated by extracellular material within the structure (Fig. 3b).

When such cultures were kept for another 36 days, fixed in situ, and analyzed microscopically, it was found that the artificial vessel structure remained essentially intact although only occasionally a continuous layer of endothelial cells could be observed. The invading tumor cells had proliferated within the vessel wall and appeared to be viable at the end of the experiment (Figs. 2d and 4b). The HT1080 cells had also infiltrated into the smooth muscle tissue of the blood vessel leaving these cells intact (Fig. 4b). In contrast to these observations, the tumor cells had completely destroyed the smooth muscle cultures after the same period of time (Fig. 2c). Very few smooth muscle cells could be found with electron microscopy (Fig. 4a). The remaining extracellular matrix consisted mainly of collagen fibers and some glycoprotein material. The fibrosarcoma cells, however, showed a viable appearance and could be regrown from such cultures (data not shown).

Further experiments showed that endothelial cells were markedly inhibitory to tissue destruction, even if they were added simultaneously or after the tumor cells (Chart 3). An inhibition of tumor cell-induced hydrolysis was detectable if the endothelial cells were added 7 days after the HT1080 cells, but the extent of protection was greater if the time between adding the 2 cell types was less. No measurable digestion of smooth muscle cell matrix proteins occurred if the fibrosarcoma cells were added 1 day after the endothelial cells. Thus, although the protective effect of the endothelial cells did not require a preexisting layer, the maximum resistance of the structure to tumor cell-induced destruction was seen when such a layer was present.

Two strains of human skin fibroblasts and the mouse embryo cell line C3H/10T½CL8 were also able to retard degradation by HT1080 cells if they were plated on top of smooth muscle multilayers 1 week prior to fibrosarcoma cells (Chart 4). These nontumorigenic cells formed monolayers before the tumor cells were added, however, the degree of protection afforded was less than that seen with a strain of bovine venous endothelial cells (V2CL2), which behaved identically to the arterial endothelial cells in this experiment. A second addition of nonradioactive rat smooth muscle cells to the labeled smooth muscle layers only weakly retarded the destructive activity of the tumor cells. None of the normal cell types used induced the release of radioactivity from the smooth muscle substrates.

The inhibitory effect of the endothelial cells was proportional to the number of cells seeded (Chart 5). In this experiment, 2 x 10^6 HT1080 cells were added to smooth muscle multilayers simultaneously with different numbers of endothelial cells. The presence of 8 x 10^5 endothelial cells caused a measurable decrease in the rate of destruction, and 4 x 10^4 endothelial cells (tumor cell:endothelial cell ratio, 5:1) markedly inhibited the hydrolytic activity of the fibrosarcoma cells.

The effect of the fibrosarcoma cells on the total amounts of extracellular matrix proteins present in smooth muscle or art-
vitalficial vessel cultures was determined directly by protein determination (Chart 6). The HT1080 cells caused a decrease in the quantities of all the extracellular matrix proteins present in smooth muscle cultures (Chart 6A). The amount of glycoprotein present in artificial vessel walls was not affected by the presence of HT1080 cells, but the cultures contained less elastin and collagen than did control cultures (Chart 6B). This result implied that the HT1080 cells interfered with the continued deposition of these proteins, since the earlier experiments had shown that there was no degradation of preexisting proteins in such cultures (Charts 2 and 3).

Cultures of smooth muscle cells or artificial vessels incubated with HT1080 cells showed decreased levels of incorporation of [3H]proline into elastin and collagen, whereas incorporation into glycoprotein(s) was relatively unaffected by the tumor cells (result not shown). Thus, although the tumor cells were ineffective in degrading preexisting proteins in the artificial vessel walls, they did inhibit the increases in the levels of these proteins which were occurring in the control cultures.

**DISCUSSION**

The complexities of whole-animal experiments necessitate that the enzymology and cell biology of tumor cell extravasation be investigated in isolated systems. The fidelity with which culture systems reflect extravasation as it occurs in vivo can at present only be assessed by morphological comparisons of in

![Chart 5](image)

**Chart 5.** Inhibition of degradative abilities of HT1080 cells by different numbers of endothelial cells. HT1080 cells (2 X 10⁵/35-mm dish) were added to smooth muscle multilayers prelabeled with [3H]proline simultaneously with 8 X 10² (O), 4 X 10¹ (△), 2 X 10⁰ (△△), 10⁰ (x), or no (•) A1.CI-1 endothelial cells. The cumulative release of radioactivity in the supernatant medium was determined every 3 to 4 days at medium change.

![Chart 6](image)

**Chart 6.** Effect of HT1080 cells on the total amounts of protein present in smooth muscle or artificial vessel wall cultures. HT1080 cells (2 X 10⁵/35-mm dish) were added to rat smooth muscle cultures alone (A) or artificial vessel wall cultures (B) 4 days after the addition of 10⁶ A1.CI-1 cells. The total amounts of glycoproteins (GP), elastin (E), and collagen (C) in cultures incubated without (x) or with (△) HT1080 cells were determined 0 or 34 days after the addition of fibrosarcoma cells.

in vitro and in vivo extravasation. In this sense, the morphological data presented here provide an essential frame of reference by which the significance of the biochemical data can be judged.

The substrates had several of the morphological characteristics of vessel walls and contained an endothelial layer anchored firmly to the underlying smooth muscle multilayer (8). Electron micrographs also showed the presence of an irregular, dense basal lamina-like structure between the 2 cell types. Although detailed studies on the intactness of the endothelial layers have not yet been performed, the existence of some gaps in the endothelium would not invalidate our approach, since such gaps do occur in vivo (20) and invasive cells penetrate "tight" endothelia both in vivo and in vitro (1, 22, 25). The structures also contained biochemically and morphologically identifiable glycoproteins, elastin, and collagen (8, 12), which are 3 of the major protein constituents of vessel walls. They therefore appeared to be reasonable substrates for the tumor cells and resembled tissues from animals because the producer cells were embedded in the extracellular matrix proteins which they had synthesized. One disadvantage of the system was that the amounts of protein present in control cultures increased throughout the experiments (Chart 6); hence, the substrates represented dynamic rather than static systems. However, our ability to measure these increases meant that quantitative data could still be obtained.

The fact that the fibrosarcoma cells rapidly established themselves in the subendothelial zone suggested that they might follow an adhesive gradient as suggested by Kramer and Nicolson (14). It was also interesting that they assumed a much flatter shape when growing in this area and often appeared to become coated by extracellular matrix material. The walling off of the tumor cells by endothelial cells following penetration appeared to mimic what has been described for extravasation in vivo (16, 24, 26).

Although the number of HT1080 cells in the subendothelial space appeared to be less than that seen in smooth muscle cultures, it was unlikely that the inhibitory effects of the endothelial cells on vessel destruction were due primarily to growth inhibition. Examination of the electron micrographs showed that considerable fibrosarcoma cell division had occurred after 36 days, and no signs of cell atrophy due to anoxia or nutrient deprivation were observed. Also, the facts that endothelial cells inhibited HT1080-induced destruction at a cell:cell ratio of 1:25 or when added up to 1 week after tumor cells argued against growth inhibition or toxicity as being completely responsible for the effects.

Nevertheless, the microscopic observations appeared to demonstrate a decreased proliferation of HT1080 cells in the subendothelial area, and these observations may have some bearing on the phenomenon of tumor cell dormancy. In this regard, it is interesting to note that Warren (24) has proposed that the subendothelium may indeed be a site where tumor cells remain dormant in the intact animal. Indeed, the connective tissue substrates appeared to increase the viability of the HT1080 cells since viable tumor cells were regrown from the cocultures on several occasions. It should also be noted that tumor cell turnover appeared to occur during the course of the experiments since floating cells, which were assumed to be of HT1080 origin, were always present in the medium before refeeding.

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The nature of the inhibitory effect of the endothelial cells on the HT1080 degradative activity is presently unknown. However, preliminary experiments have shown that medium conditioned by endothelial cells can inhibit the plasminogen activator activity in the HT1080 supernatant without the need for cell-cell contact (6). Since plasmin production has been found previously to play a significant role in the degradation of extracellular matrix by HT1080 cells (9), the lowering of plasminogen activator activity may well influence the rate of tissue destruction. The use of these artificial vessel walls may therefore increase our knowledge of tumor cell extravasation and provide more information on interactions between normal and tumor cells in the animal.

REFERENCES

Fig. 1. Effect of human fibrosarcoma cells on the total amounts of extracellular matrix material present in smooth muscle or artificial vessel walls. HT1080 cells (2 x 10⁴/35-mm dish) were seeded onto rat smooth muscle layers or artificial vessel walls, and the cells were lysed by the addition of 0.25 M NH₄OH at the indicated times after seeding. The residual matrix was then stained with Coomassie blue before photography. Top row, cultures containing rat smooth muscle cells only; second row, as above but seeded with HT1080 cells; third row, cultures containing artificial blood vessel walls; bottom row, as above but incubated with HT1080 cells.

Fig. 2. Human fibrosarcoma cells were seeded on cultures of smooth muscle cells (a, c) or artificial blood vessel walls (b, d), fixed in situ, and processed for electron microscopy. Within 24 hr after addition, tumor cells (closed arrows) attached to the surface of smooth muscle cells and some were spread out (a), whereas on the artificial blood vessel some of the HT1080 cells (closed arrows) penetrated the endothelium and were seen to invade the subendothelial space (b). When such cultures were fixed 36 days later, the smooth muscle cultures had been destroyed by overgrowing tumor cells (c). In contrast, the structure of the artificial blood vessel was more intact with the endothelial cells on top (open arrows), while the tumor cells had invaded and proliferated within the vessel wall. × 1,250.

Fig. 3. a, HT1080 cells were seeded on smooth muscle cell cultures (SMC) and fixed in situ 24 hr later. This micrograph shows 2 tumor cells, which developed a primitive intercellular junction (arrow), attached to the upper surface of smooth muscle cells. The fibrosarcoma cells showed their characteristic morphology including numerous plasma membrane projections. No invasion of the smooth muscle cell tissue by the tumor cells occurred within this period of time. Note the extracellular material between the smooth muscle cells. × 10,400. b, tumor cells (TC) were seeded on the artificial blood vessel and fixed in situ as indicated in a. The fibrosarcoma cells had penetrated the endothelial cell layer which shows an area of noncontinuity. Beneath the endothelial cells (EC) is a basement membrane-like structure of extracellular matrix material (open arrows). The tumors, which contain many structurally normal mitochondria, are surrounded by extracellular material. Smooth muscle cells show their characteristic thin and flat morphology. × 10,600.

Fig. 4. a, smooth muscle cell cultures (SMC) were fixed 36 days after tumor cell addition. These cultures were overgrown and completely destroyed by the fibrosarcoma cells. The remaining smooth muscle cells showed many lysosomal bodies and the majority of the extracellular material had been degraded by the HT1080 cells leaving a few collagen fibers and some glycoprotein material. × 10,480. b, this micrograph shows an artificial blood vessel fixed 36 days after cocultivation with HT1080 cells. These cultures occasionally showed endothelial cells (EC) on top of the structure, but most of the surface was covered by tumor cells (TC). HT1080 cells appeared viable with well-organized cytoplasm and nuclei; however, some of them showed a few lysosomal bodies. The fibrosarcoma cells were seen to have invaded into the smooth muscle tissue of the artificial blood vessel; the surrounding smooth muscle cells, however, showed their typical flat appearance and were coated by extracellular material. × 6,500.
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