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Proenzyme Therapy of Cancer*

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Abstract. *Proteases and their inhibitors have long been investigated in numerous tumor systems, and at the tumor growing front, their balance has been universally found to be shifted towards higher proteolytic activities. However, out of many promising serine and metalloproteinase inhibitors, none are included in cancer treatment regimens at present. The current search for active antiproteolytic compounds is in contrast to the classical approach developed by John Beard, who suggested treating advanced cancer by fresh pancreatic extracts whose antitumor activity was based on their proteolytic potential. We followed John Beard's recommendations by using purified pancreatic proenzymes/enzymes, trypsinogen/trypsin (TG/TR), chymotrypsinogen/chymotrypsin (CG/CH) and amylase (AM). The mixture of these enzymatic activities produces potent antimetastatic and antitumor effects in cellular, animal and human systems. The treatment of cultured tumor cells with TR and CH at nanomolar concentrations, comparable to those achieved in the blood of the patients, causes complete arrest of the directional movement of metastatic cells. Conversely, the same treatment of normal cells results in enhanced motility and an accelerated closure of the gap created in cell monolayers. Further, treatment of cells with serine proteases results in the formation of cellular 3-dimensional structures such as lamellae, cell streams and aggregates. In some cell types, the aggregates are compacted via cadherin-based cell-cell communication systems and form compact spheroids. In the highly metastatic cells with lower cadherin expression, the ability to form spheroids also diminishes. Tumor cells unable to form spheroids when treated with proteases are subject to elimination by apoptosis. In contrast, a large proportion of cells that form spheroids remain viable, although they are metabolically suppressed. Protease-treated tumor cells contain a disrupted actin cytoskeleton and exhibit a loss of front-to-back polarity. We hypothesize that the provision of zymogens, rather than the enzymes, was of crucial importance to the clinical effectiveness in the human trials conducted by Beard and his co-workers. The precursor nature of the active enzymes may offer protection against numerous serpins present in the tissues and blood. Experimental evidence supports the assertion that the conversion from proenzyme to enzyme occurs selectively on the surface of the tumor cells, but not on normal cells. We believe that this selectivity of activation is responsible for the antitumor/antimetastatic effect of proenzyme therapy and low toxicity to normal cells or tumor host. Elevated levels of endostatin and angiostatin appear in the blood of TG/CG/AM-treated tumor-bearing mice, but not in tumor mice treated with the vehicle alone or in proenzyme-treated tumor-free mice. These findings support the conclusion that proteolysis is the active mechanism of the proenzyme treatment. Future studies will focus on the molecular mechanisms of the proenzyme therapy including the identification of molecular target(s) on the tumor cells. In conclusion, we have discovered that proenzyme therapy, mandated first by John Beard nearly one hundred years ago, shows remarkable selective effects that result in growth inhibition of tumor cells with metastatic potential.*

Nearly one hundred years ago, John Beard, a professor of comparative embryology at the University of Edinburgh, UK, made an observation that the pancreas develops in most vertebrates at the time when the placenta begins to slow its rate of growth. He hypothesized that enzymes produced by the developing pancreatic gland curtail trophoblastic invasion and suggested that pancreatic extracts should have a similar inhibitory effect on invasive tumors (1). Subsequently, pancreatic enzyme extracts were employed by several of his contemporaries (Table I) and were found to effectively abrogate cancerous growth in advanced cancer patients (2-15). Nevertheless, diminishing results reported by others (16, 17) caused his method and teachings to fall into disrepute and they were gradually

*Preliminary report dedicated to the Memory of John Beard (1857 - 1924)

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Table I. *Curative treatments of cancer patients treated with pancreatic extracts, 1905-1909.*

Author	Year	Tumor	Ref.
Beard, J.	1905	theoretical basis	1
Cleaves, M.A.	1906	ca. of the rectum	2
Rice, C.C.	1906	ca. of larynx	3
Wiggin, F.J.	1906	fibrosarcoma of tongue	4
Golley, F.B.	1906	ca. of colon and uterus	5
Scott, C. C.	1907	ca. of caecum	6
Campbell, J.T.	1907	ca. of tongue	7
Goeth, R.A.	1907	breast ca.	8
Dupuy, H.	1907	epithelioma of larynx	9
Cutfield, A.	1907	ca. of pancreas	10
Donati, M.	1907	testicular ca.	11
Marsden, A.	1908	ca. of cervix	12
Lambelle, F.W.	1909	sarcoma of the jaw	14
Golley, F.B.	1909	follow up of ref. 5	13

forgotten. In several excellent compilations of the history of cancer science and treatment (18-21), John Beard's ideas and clinical results concerning enzyme therapy of tumors went without mention.

In modern times, pancreatic extracts are used to treat chronic or acute pancreatic insufficiency due to pancreatic disease or cystic fibrosis (22, 23). These oral preparations contain variable amounts of proenzymes and active pancreatic enzymes, which are formulated to pass through the gastric environment before they are deposited in the intestines (24). Active trypsin, chymotrypsin and other proteases are also components of the commercially available enzyme mixtures Wobe-Mugos E and Phlogenzym (Mucos Pharma GmbH, Geretsried, Germany). The latter products were tested for treatment of neoplastic, autoimmune or viral diseases (25-27). Importantly, the Wobes-Mugos E preparation, when provided orally, results in measurable relief from cancer disease- and therapy-associated symptoms and causes a small but significant extension of survival. In clinical trials involving hundreds of breast cancer (28), colorectal cancer (29) and multiple myeloma (30) patients, an improvement was noted in all adverse complications except infections. Several mechanisms, including suppression of cytokine levels (31) and enhancement of polymorphonuclear leukocyte-mediated cytotoxicity (32), were suggested to be responsible for the adjuvant effectivity of the Wobe-Mugos E enzyme mixtures. Significant reduction of radiation therapy side-effects by oral hydrolytic enzymes was also reported (33). Although the enzymes are usually given in massive amounts (34), some authors contend that none (35) or only a small fraction (0.002-0.0025%; 36) of the initial oral intake of active enzymes appear to be absorbed into the blood stream. It is possible that entry of these exogenously supplied enzymes into the blood could follow the still controversial path of endogenous digestive enzymes, as proposed by the conservation theory (37, 38). Barring difficulties with absorption, the active proteases are likely to be rapidly rendered inactive in blood plasma by numerous serpins (39, 40).

We have considered the original observations of Beard (15) and respected his insistence that the pancreatic extracts must be fresh if they are expected to retain antitumor activity. Unknown at the turn of the 20th century was the fact that most of the pancreatic enzymes are secreted in a proenzyme form and then sequentially activated in the duodenum *via* the action of enterokinase (41). The crucial hypothesis that proenzymes and not the activated enzymes were the pivotal components of Beard's preparations was, for the first time, recognized and tested on tumor-bearing animals by Trnka *et al*. (42). Here, we report that a mixture of trypsinogen, chymotrypsinogen and amylase, approximately in concentrations recommended by Beard, provides for a potent antitumor, anti-invasive and anti-angiogenic agent. One of the major effects of the proenzyme/enzyme mixture at the cellular level is the inhibition of tumor cell migration. In addition, we discovered that continuous exposure of tumor cells to low concentrations of trypsin/chymotrypsin (TR/CH) or their proenzymes (trypsinogen/chymotrypsinogen; TG/CG) leads to the formation of cellular aggregates similar to the previously described multicellular spheroids (43, 44). The majority of *in vitro* experiments were conducted with active enzymes, however, we demonstrated that metastatic tumor cells have the potential to convert proenzymes into active enzymes. We propose that these protease-elicited changes, observed either *in vitro* or *in vivo,* are responsible for the antitumor and antimetastatic effects in tumor-bearing animals and humans, resulting in the observed extension of life in both. The possibility that natural or pharmacologically-induced excessive tumor pericellular proteolysis could be used in the fight against cancer has been postulated by Reijerkerk *et al*. (45). This is supported by paradoxical findings that overexpression of matrix metalloproteinase-9 (46) or of prostate-specific antigen (47) result in an improved prognosis for some tumor patients. In an effort to combine historical and recent observations, we were guided by the following hypotheses assumptions: (i) protease proenzymes are resistant to inactivation by protease inhibitors, (ii) the activation of proenzymes occurs exclusively at the tumor cell membrane, (iii) active serine proteases attack as yet unknown tumor cell surface molecule(s), and (iv) positive historical and recent clinical outcomes are based on the proteolytic action of the introduced (pro)enzymes. In this preliminary communication, we report that pancreatic proenzymes/

enzymes constitute an effective tool to combat metastatic cancer. We support this proposal with several novel observations concerning the behavior of tumor cells and *in vivo* tumors under protease stress. We and others have begun detailed studies on some of these observations, which will be reported elsewhere.

Materials and Methods

Materials. The following proenzymes and enzymes were all of bovine pancreas origin: trypsin (Worthington Biochemical Corp. Lakewood, NJ, USA, code TRLS), chymotrypsin (Worthington, Code CDI), trypsinogen (Worthington, Code TG and Sigma, St. Louis, MO, USA; T1143), chymotrypsinogen A (Worthington, code CGC and Sigma, C4876). Alpha-amylase (Type IIA from *Bacillus* species) was purchased from Sigma. CaspAce FITC-VAD-FMK cellular pre-apoptotic marker was from Promega Corp. (Madison, WI, USA). Anti-pan-cadherin, anti-F-actin and anti-G-actin antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Growth Factor Reduced BD Matrigel Matrix was from Matrix Biosciences (Bedford, MA, USA). Murine angiostatin polyclonal antibody and the Competitive Mouse Endostatin Immunoassay were from Neogen Corporation (Lexington, KY, USA).

Cell culture and treatment. The following cell lines were obtained from ATCC (Manassas, VA, USA): MCF-7 and MDA-MB-231 (breast carcinomas), MG-63 and U-2OS (human osteosarcomas), WiDr (human colorectal carcinoma), LC-540 (rat Leydig tumor cell line), HEK293 (adenovirus 5-transformed human kidney epithelial cell line), PC-3 (human prostate carcinoma), MDCK (canine kidney normal epithelial cells) and B16F10 (murine melanoma). Primary human osteoblasts NHOst were from BioWhittaker, Inc. (Walkersville, MD, USA); OBPU is a continuous cell line derived in our laboratory from a human bone sample provided by Dr. R. Leggon (Penn State Geisinger Health System); NRK-52E rat kidney epithelial cells were a gift from Dr. S. Nyquist (Bucknell University). All cell lines were maintained in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal calf serum and penicillin/streptomycin (5,000 U and 5,000 μg /ml, respectively). The cells were maintained at 37˚C in 5% carbon dioxide and 95% air. Treatment with enzymes and/or proenzymes was conducted in one of the following serum-free media: VP-SFM (Invitrogen) and DMEM/F12, as described in the legends.

Cell growth and proliferation. Cell growth was assessed by tetrazolium-generating assay (XTT assay; Roche Diagnostics GmbH, Mannheim, Germany), and by protein content (BCC assay, Pierce Biotechnology, Inc., Rockford, IL, USA). Cell proliferation was estimated by means of bromodeoxyuridine incorporation and detection assay (RPN256 cell proliferation assay; Amersham, Piscataway, NJ, USA) and by incorporation of methyl-^{3}H-thymidine (Moravek, Brea, CA, USA). All these procedures were conducted according to the recommendations of the manufacturers and standard methods.

Apoptosis. CaspACE FITC-VAD-FMK is a cell-permeable fluorescein-conjugate of an inhibitor of activated caspases. The cells were treated with enzymes or proenzymes as described in the figure legends. Because of the semi-adherent state of some of the 3-dimensional structures and spheroids in enzyme-treated cultures, the free-floating cells in media were combined with the cells that were trypsinized by trypsin/EDTA. The cells were centrifuged (400xg for 5 min) and suspended in DMEM/F12 serum-free media to a density of approximately 10^6 cells/ml. The FITC-VAD-FMK was added to a final concentration of 10 mM and cell suspensions were incubated at 37˚C for 20 min. The cells were fixed with 4% formaldehyde for 30 min and centrifuged as above. The cellular sediments were resuspended in 5 ml of Vectashield with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) and the suspension mounted on microscopic slides. Identical images were obtained with DAPI and FITC filters and analyzed visually.

Immunocytochemistry. Fluorescent immunocytochemistry was conducted using one of the following three approaches: cells were cultured on 12-mm circular glass 1.5 coverslips (Fisher, Pittsburgh, PA, USA), Cover-Well imaging chamber gaskets (Molecular Probes, Eugene, OR, USA) and directly on Optical Bottom 96-well plates (Nunc, Rochester, NY, USA). The cells were seeded and grown to a desired confluency and successively washed twice with phosphate-buffered saline (PBS) and once with serum-free medium. The cells were treated with enzymes or other agents for a specified time and subsequently washed three times with PBS. The cells were fixed with either 3.75% formaldehyde or with ice-cold methanol for 10-15 min. For visualization of the intracellular antigens, the fixed cells were permeabilized by 1-min incubation in 0.1% Triton/0.05% Tween 20 or by an exposure to cold acetone for 5 sec. The cells were rehydrated with PBS for 10 min at 37˚C. The PBS was replaced with 1% BSA for 1 h at 37˚C to block non-specific binding. The cells were treated with 30-100 μl of a primary antibody in an appropriate dilution with 1% BSA containing 50 ng/ml of RNAse A for 4 h at 37˚C. The antibody solution was removed and the cells were washed 5 times for 5 min each with PBS. A solution of fluorescein isothiocyanate-conjugated secondary IgG antibody (Sigma), diluted 1:200 in 1% BSA, was added and the cells incubated for 1 h. The cells were finally washed 5 times for 5 min each and mounted on glass slides using Vectashield mounting media with or without DAPI. The cells were observed using fluorescence and/or differential interference contrast (DIC) microscopy.

The cellular aggregates described above were either free floating or loosely adhered to the substrate. Thus, it was difficult to submit these cultures to a standard procedure such as immunohistochemistry, which requires multiple washings and incubations. Several approaches were tested to solve this problem, as described below. The incubation of cells with Cell-Tak (BD, Bedford, MA, USA) for 20 min prevented the loss of 3-dimensional structures; unfortunately, Cell-Tak interfered with the staining by antibodies. In some cases, 3-dimensional structures were detached and stained in suspension and then plated on the microscopic slides coated with Cell-Tak. Our second approach was to use Molecular Probe manufactured cell growth platforms (Press-to-Seal Silicone Isolators with adhesive; Molecular Probes) followed by washing and labelling within protected surfaces provided by these devices. Finally, cells and cellular structures were collected into microcentrifuge tubes and processed as non-adherent cells before placing the structures on the polylysine-coated slides and mounting. Only the latter method enabled routine completion of the necessary processing steps, although it was not possible to preserve all of the original natural structures.

Invasion assays. Matrigel extracellular matrix was used to assess the ability of tumor cells to grow and invade semi-solid gels. Prior to gelling, the Matrigel was mixed with the desired level of proenzymes, whereas the controls received the appropriate amount of vehicle alone. 0.15 ml of Matrigel-proenzyme mixture was added into flat bottomed 96-wells and allowed to gel for 4 h at 37˚C. The solidified gel was washed before the cells were seeded on top of the gels in a 150 ml volume of serum-free media. The cell penetration into the Matrigel was monitored by means of inverted phase contrast microscopy. In concurrent experiments, the proenzymes were added together with the cells on top of the proenzyme-free gels or incorporated directly into the gels.

Treatment of transplanted and induced murine tumors. Female C57Bl6 mice (average weight 28 g) were housed in a 12/12-hour photoperiod environment with free access to food and water. B16F10 murine melanoma cells were maintained as ascites in the peritoneal cavity of mice or in *in vitro* cultures, as described above. For tumor inoculation, the intraperitoneal or tissue culture cells were resuspended into Hanks solution at a density of $1x10^6$ in 0.1 ml. Tumor cells (1-2 x 10^6) were transplanted intradermally into the left flank of the mice. On day 10 following transplantation, the incipient tumors were surgically removed under anesthesia. Tumor cell-transplanted mice were distributed into groups of 10 and treated the same day with trypsinogen, amylase and pancreatic extract (see Results). Drugs were provided either as single agents or in various mixtures. The final concentrations of drugs were adjusted for a murine system from the dosages calculated for humans, as recommended by Beard (15). The basic dosage consisted of 750 BAEE units (as defined by the manufacturer) trypsinogen and 6.66 units amylase per gram of mouse in a total volume of 0.1 ml. Various multiples of these concentrations were tested as indicated in the Results. Injections of the (pro)enzymes were delivered subdermally or intramuscularly into the opposite flank from the primary tumor. Controls included groups of tumor-bearing mice with or without operation. Preliminary experiments indicated lack of any noticeable side-effects due to daily application of the (pro)-enzymes, even for a period over three months.

Methylcholanthrene-induced tumors were induced by administration of 3-methylcholanthrene diluted in olive oil (1:2 dilution). 0.2 ml of the dissolved carcinogen (400 μg of methylcholanthrene) was applied by subcutaneous injection into the right flank of a mouse. Two additional injections were applied to the same area during the following two days (each mouse thus received 1.2 mg of methylcholanthrene). Tumors appeared on days 37-44 after the first application. The tumor-bearing animals were selected on day 45 and divided into three experimental groups, each including 10 mice and one group of 8 mice (control). The control group was treated with saline instead of proenzyme compositions. Treatment consisted of three different combinations of trypsinogen and amylase, prepared in a similar way as described in the B16F10 experiments above. The mice were treated by injections into an area distant (left flank) from the developing tumor. The indicated amounts of agents were delivered subcutaneously in a total volume of 0.1 ml, once a day. The tumor size was measured twice weekly and the surviving mice checked every day.

Endostatin immunoassay. Female C57BL6 mice (14 weeks old) were injected subcutaneously with 1 x 10^6 B16F10 cells. On day 12 post-tumor inoculation, the control (tumor-free) and tumor-bearing animals were treated with a mixture of trypsinogen, chymotrypsinogen and amylase (see Figure 17 and the Results) at 0, 5, 8 and 24 hours prior to blood collection. The mice were anesthetized (ketamine/xylazine) and blood obtained by cardiac puncture. The blood was collected into microfuge vials containing a sufficient amount of heparin to obtain a final concentration of 50 U/ml of blood. The blood was centrifuged at 400xg and plasma frozen at -70˚C until used in the Competitive Mouse Endostatin Assay system.

Western blotting. Plasma samples, tumor initiation and tumor treatments were prepared as described for the endostatin determination above. Plasma proteins were separated by means of SDS-PAGE in a 10% polyacrylamide Tris-glycine-SDS gel. Following electrophoresis, the proteins were transferred onto nitrocellulose membranes (BioRad) in SDS-free Tris-glycine buffer at a constant voltage (80 V) for 1 h. The blot was blocked using 10% dry non-fat milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) for 1 h with agitation. The blot was washed 5 times for 5 min each with TBST and then exposed to polyclonal antibodies, derived in rabbit against angiostatin of mouse origin (1:4000; Neogen Corp.), in 1% dry milk/TBST overnight at 4˚C. The blot was washed 5 times for 5 min each with TBST and exposed to peroxidase conjugated anti-rabbit IgG antibody (Sigma) diluted 1:5000 in 1% dry milk/TBST for 30 min at room temperature. The ECL™ chemiluminescent detection system (Amersham Life Sciences, Piscataway, NJ, USA) was used for visualization of antibody-positive bands and the blots were exposed to Hyperfilm™ for 5 min.

Results

Trypsin and chymotrypsin cause spheroid formation in cell cultures. The continuous presence of a 1:1 (w/w) mixture of trypsin and chymotrypsin (TR/CH) in serum-free media of subconfluent HEK293 cells led to morphological changes, culminating in the formation of spheroids (Figure 1). We recognized a uniform sequence of cellular reorganization which precedes the formation of spheroids in various tumor and/or normal cells treated with increasing concentrations of proteases. Using subconfluent HEK293 cells (Figure 1A) as an example, this progression consisted of alignment of the cells along their longitudinal axes into cellular streams (Figure 1B), followed by a gathering of the cells into tighter 2-dimensional "flattened" groups and lamellae (Figure 1C). The cell streams, lamellae and cell groups collected additional surrounding cells and formed distinct cords (Figure 1D), which connected to increasingly 3-dimensional cell groups. The cords continued to be drawn towards the cell groups, became disconnected (Figure 1E), and the cell groups converted into 3-dimensional loosely-attached spheroids (Figure 1F). Each of the steps represented protease concentration-dependent morphological progression. Thus, prolonged treatment of the cells with lower enzyme concentrations did not necessarily lead to the formation of a higher order of structures. In some cells, such as MCF-7, we observed a gradual collecting of smaller spheroids into huge conglomerates simply by continuing culture at high spheroid-forming protease levels. At concentrations of enzymes higher

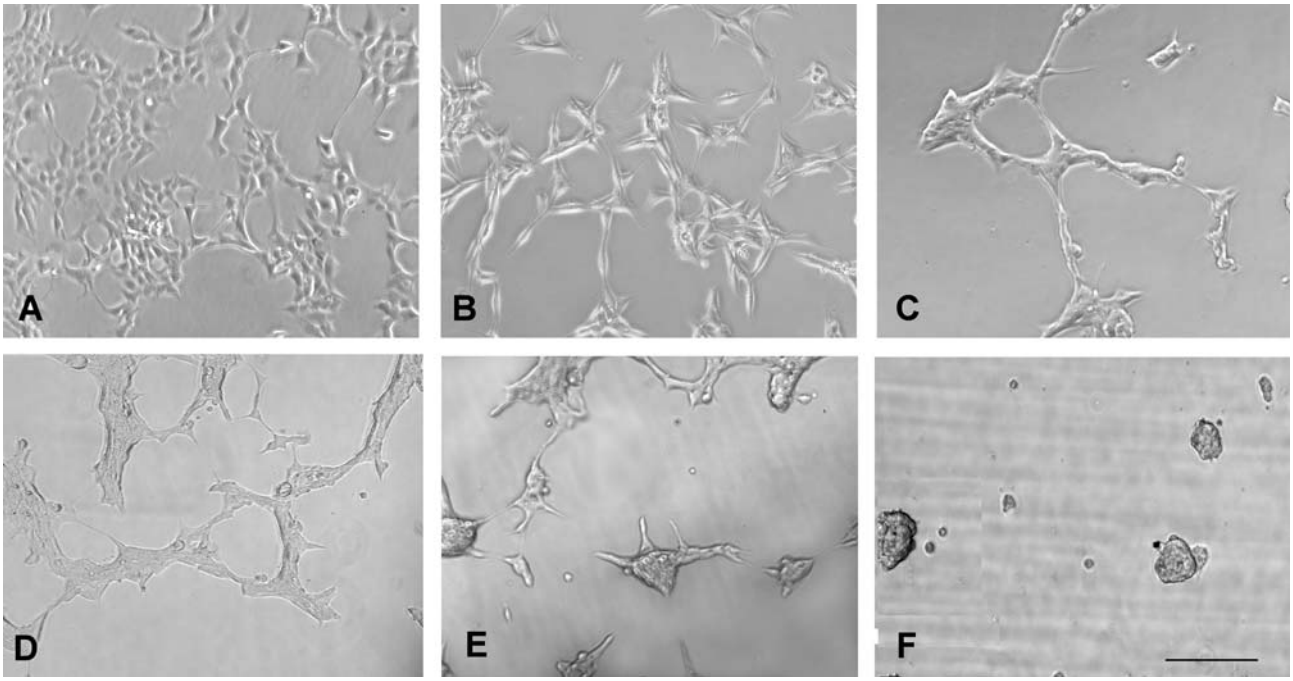

Figure 1. *Protease-mediated formation of multicellular structures and spheroids from subconfluent cultures. Human kidney AD5-transformed cells HEK293 were seeded into 96-well plates (10,000 cells/well). Twenty-four hours later, the cells were washed two times with serum-free media and 0.3 ml of DMEM/F12 containing trypsin and chymotrypsin was added to each well. The final concentrations of each, trypsin and chymotrypsin, in ng/ml, were as follows: 0 (A), 100 (B), 200 (C), 400 (D), 800 (E), 1600 (F). The cells were photographed at the end of 24-hour incubation using inverted phase contrast microscopy. Scale bar indicates 100 μm.*

than 20 μg/ml, the spheroids appeared to diminish in size and single non-viable cells broke out from the spheroids. The described sequence may encompass a 24-h period; however, the initial changes manifested within a few hours, especially at high enzyme levels. The concentration of serine proteases required for spheroid formation depends on the type of tumor cell; however, most of the neoplastic cells formed spheroids between 100 ng/ml and 3200 ng/ml of each TR and CH (Figure 2A). Two of the tumor cell lines tested, U2OS human osteosarcoma and MDA-MB-231 human breast carcinoma, were unable to form spheroids at any of the concentrations up to 12.8 μg/ml of TR and CH (total of 25.6 μg/ml protease protein). Other conditions, such as the absence of glucose and oxygen, appeared to enhance the formation of the spheroids (unpublished results). Most normal primary and non-tumorigenic but immortalized cell lines were unable to form spheroids (Figure 2B). Among normal cells, MDCK as well as NRK52E proved to be an exception and formed spheroids. Nevertheless, instead of gathering in spheroids, many normal cells balled up, detached and underwent anoikis.

The initial cell density significantly influences the level of enzymes needed to induce changes leading to the formation of spheroids. At least twice as much enzyme is required to produce spheroids from confluent cultures as compared to subconfluent cells (Figure 3). Spheroid formation is dependent on the enzymatic activity of proteases as the addition of a 2-fold molar excess of soybean trypsin inhibitor prevented all cellular responses described above (Figure 4). Equimolar levels of an inhibitor and enzymes led only to a delay, not the abolition of spheroids. There is an unequal, although additive, contribution of trypsin and chymotrypsin towards spheroid formation. Figure 5 depicts the formation of spheroids upon addition of 100 ng/ml of TR and 100 ng/ml CH, not upon addition of 200 ng/ml of each of the enzymes separately. We conclude that protease-mediated formation of spheroids occurs in both normal and neoplastic cells, although in the latter it is commonly obtained at considerably lower protease levels. The spheroid forming capacity appears to depend on at least two properties of the cells: strength of the cell-matrix attachment and a type of cell-cell interaction.

Proliferation and metabolism in protease-treated cell cultures. The mid-confluent cultures of MCF-7 (Figure 6A), MG-63 (Figure 6B) and B16F10 (Figure 6C) tumor cells were subjected to treatment with a range of enzyme concentrations. The proliferative potential of cells was studied *via* incorporation of 3H-thymidine (Figure 6A). The metabolic activity was

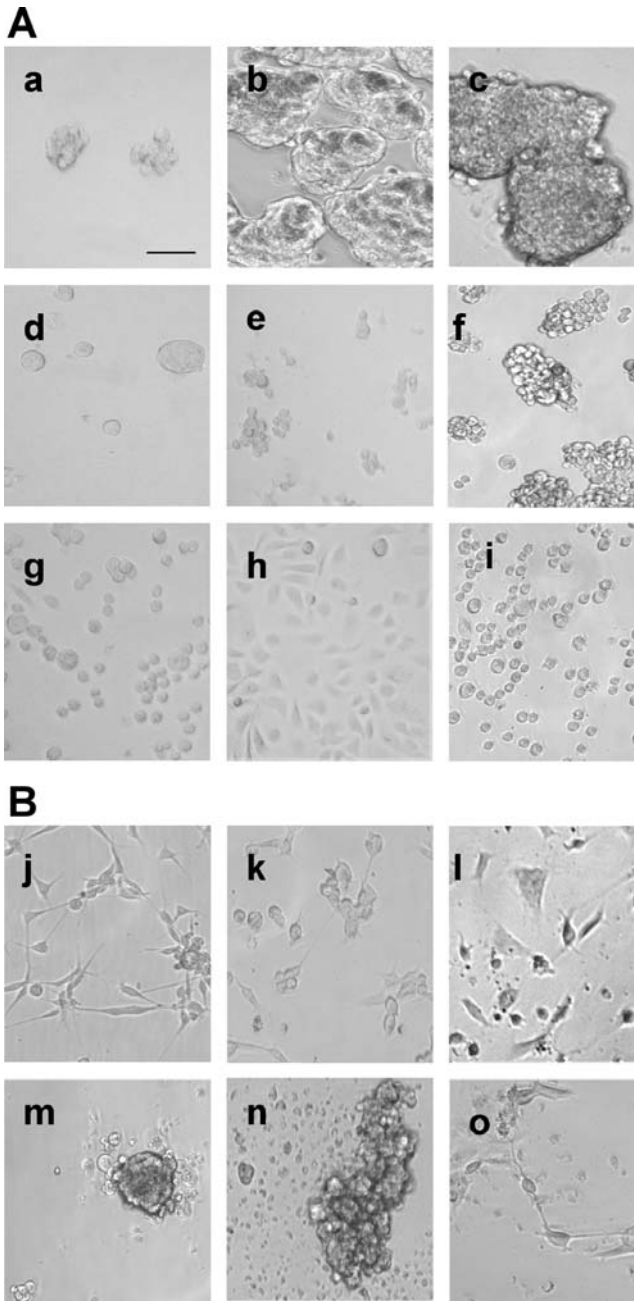

Figure 2. *Spheroid formation by various cell lines following a treatment with trypsin/chymotrypsin. Cultured cells were treated with a range of TR/CH concentrations as described in Figure 1. The photographs were taken at the lowest TR/CH levels at which spheroids were formed. Cell designation is followed by a spheroid-forming level of TR/CH in ng/ml: (A) Transformed and tumorigenic cell lines: [a] MG-63. 400; [b] MCF-7. 400; [c] JEG-3. 800; [d] WiDr. 400; [e] LC450.400; [f] B16F10. 1600; [g] PC-3. 800; [h] U-2OS. 12800; [i] MDA-MB-231. 12800. (B) Primary cells and immortalized cell lines: [j] HFF. 12800; [k] OBPU.12800; [l] HuOB.12800; [m] MDCK. 3200; [n] NRK52E. 1600; [o] MCF10A. 12800. The bar in image Aa is 50 μm and indicates identical magnification in all the subsequent images.*

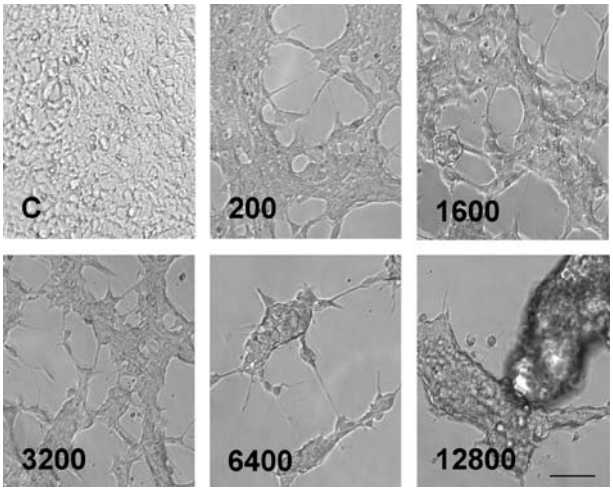

Figure 3. *Protease-mediated formation of multicellular structures and spheroids in confluent cultures of HEK292 cells. 96-well plates were seeded with 20,000 cells and the cultures maintained until confluency stage with serum-rich media. At confluency, the cells were washed and enzymes added as described in Figure 1. The final concentrations of each enzyme (TR and CH) is indicated in the figures in ng/ml. The cultures were photographed 24 hours after addition of enzymes Scale bar is 50 μm.*

evaluated by means of the XTT assay (Figure 6B) and cell survival by total protein content (Figure 6C). Both the rate of proliferation and the metabolic processes mediated by the mitochondria-based respiration were inhibited at the end of the 24-h incubation period. The inhibition of growth or metabolism was protease concentration-dependent. Remarkably, the total protein levels in the control and enzyme-treated cultures did not change significantly during the first 24 h (and 48 h, results not shown), suggesting a lack of significant cytotoxicity and apoptosis in subconfluent cultures. The response pattern of a variety of cell types in terms of their proliferative potential, metabolic activity and total protein capable of forming spheroids was similar to the examples shown in Figure 6.

Survival of protease-treated cells depends on cell density. Metabolic studies (Figure 6) showed that the process of spheroid formation is not accompanied by widespread induction of cell death. It occurred to us that the gathering of cells into cell streams and spheroids could be an attempt by cells to survive. By the same reasoning, solitary cells, unable to meet with other cells, should not survive in the presence of proteases. To test this hypothesis, we used a VAD-FMK-FITC marker to identify pre-apoptotic cells with activated caspases. The results showed that a majority of solitary MCF-7 cells, lacking any cell-cell contact at the time of proteases additions, entered a pre-apoptotic stage in the presence of TR/CH (Figure 7A). In contrast, cells that grew in 2-dimensional cell groups at the time of protease addition formed spheroids, which consisted largely of non-apoptotic cells. Some of the cells labelled with the VAD-

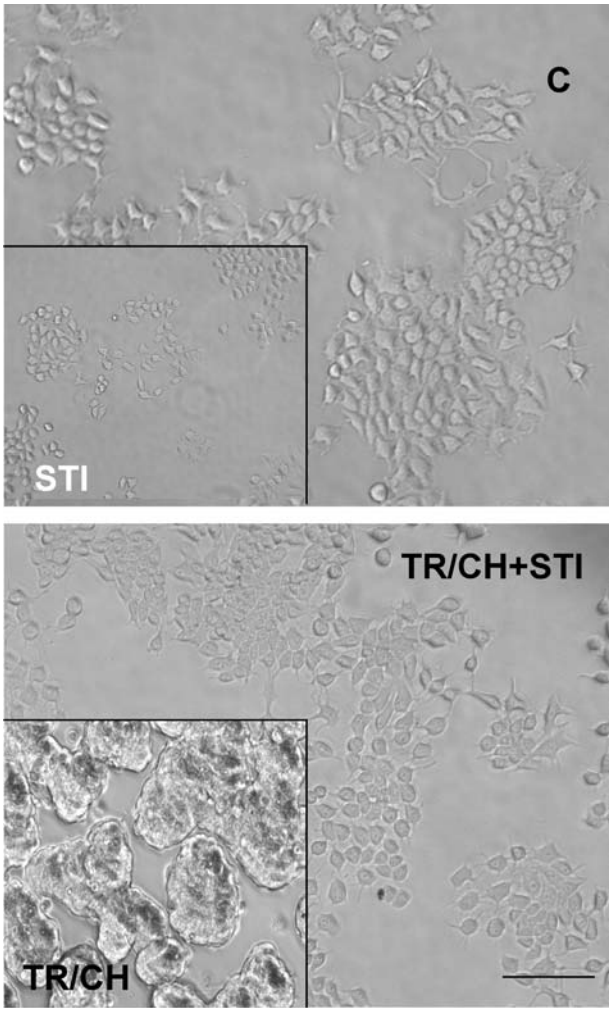

Figure 4. *The spheroid formation is dependent on the enzymatic activity of TR/CH. Subconfluent MCF-7 cells (C, control) were treated with 400 ng/ml TR/CH for 24 hours. Simultaneous addition of enzymes and excess STI (2000 ng/ml; TR/CH+STI) to the cultures prevents formation of secondary cellular structures including spheroids. Additional controls: MCF-7 cells cultured with STI alone (STI), and MCF7 treated with TR/CH alone (TR/CH; 400 ng/ml) are shown in the inserts. Scale bar is 50 μm.*

FMK-FITC complex either lacked or were only weakly labelled with DAPI (cells identified by arrows in Figure 7). In control MCF-7 cultures treated with high concentrations of cycloheximide and actinomycin D, the apoptosis was induced equally in sparse and solitary cultures (results not shown). The experiment shown in Figure 7B was conducted with MDA-MB-231 cells, a highly metastatic cell lacking significant cell-cell interactions even at confluency and an inability to form spheroids. As predicted, these cells were unprotected in the presence of proteases. As a result, the number of cells marked with VAD-FMK (apoptotic index) were high in enzyme-treated solitary as well as sub-confluent cultures.

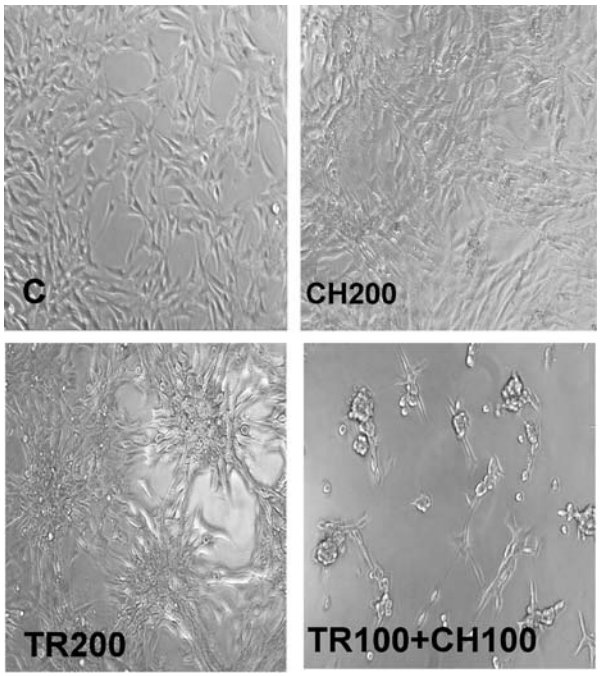

Figure 5. *The effects of TR and CH are synergistic. Subconfluent MG-63 cells were subjected to a treatment with trypsin (TR; 200 ng/ml) and chymotrypsin (CH; 200 ng/ml) alone and in combination (100 ng/ml each), as indicated in the figures. The cells were photographed using phase contrast microscope 42 hours after addition of the enzymes.*

Proteases inhibit migration of tumor cells. The effect of proteases on directional migration of tumor cells was evaluated by means of a cell monolayer "wound" assay. We found that exogenous proteases inhibit movement of U2OS osteosarcoma cells at very low concentrations of combined trypsin and chymotrypsin proteases (25 - 100 ng/ml; Figure 8). The inhibition of tumor cell migration was concentration-dependent. The inhibitory response to low concentrations of exogenous proteases occured regardless of a given tumor cell's ability to form protease-induced cellular spheroids. In contrast, the closure of the wounds in monolayers of normal cells was invariably enhanced in the presence of the same concentration of proteases. This enhancement was modest in some cells, *i.e.* MCF710A, or pronounced, *i.e.* NRK52E (Figure 9). Additional normal cells exhibited similarly weak (*i.e.* HFF, OBPU) or intense (*i.e.* MDCK) responses. We conclude that normal and tumor cells exhibit opposite migration responses to exogenous proteases.

Proteases abolish microfilament polarity of the migrating tumor cells. The cells in sparse cultures and those at the edge of the wounded monolayers are front-to-back polarized as they migrate to colonize open space. Since the lamellipodium is the major site of actin polymerization and branching, we

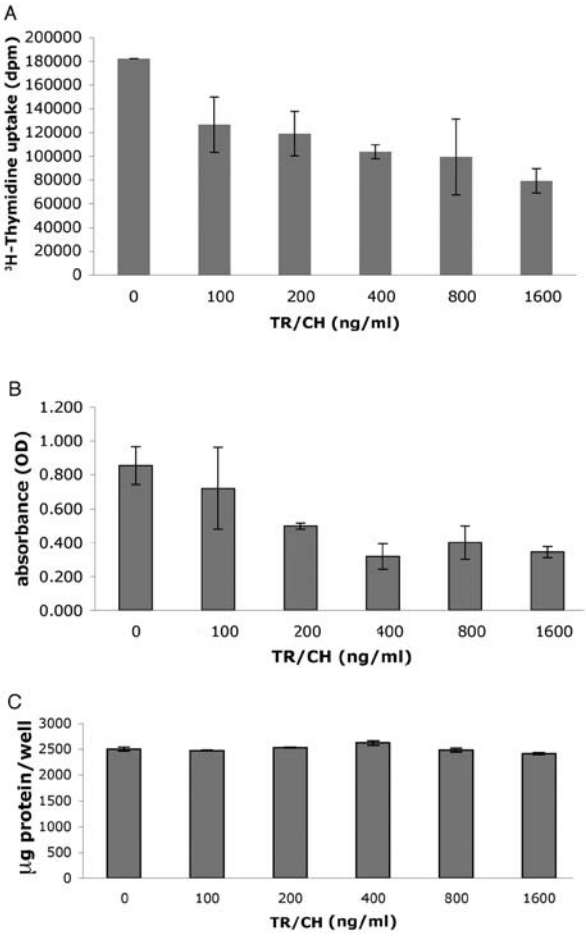

Figure 6. *DNA synthesis, mitochondrial function and total protein in spheroid-forming tumor cell cultures treated with proteases. ^{3}H-TdR incorporation (A), XTT (B), and BCA total protein (C) assays were conducted as described in Materials and Methods. All cells tested, MCF-7 (A), MG-63 (B) and B16F10 (C), were approximately 50% confluent at the time of the TR/CH addition. All enzyme treatments lasted for 24 hours: ^{3}H-TdR (2 mCi/ml) incorporation was followed during the last 4 hours, XTT assay was conducted during the last 3 hours, and protein was determined at the end of the 24-hour culture period.*

examined whether the inhibitory effect of enzymes on the migration of tumor cells in wound-healing experiments is underlined by the inability of cells to form microfilaments at their leading edge. Phalloidin-RITC fluorescence microscopy revealed profound differences in the patterns of polymerized actin between control and enzyme-treated cells. Tumor cells located at the margins of the wound and treated with enzymes (Figure 10B-C) lost the distinct directional lamellipodia exhibited by the control cells (Figure 10A). Instead, the appearance of multilateral microfilament polymerizations became evident with increasing enzyme level treatments. In some cases, the leading edge formed at the rear of the cell facing the bulk of the monolayer, indicating deregulation of the cellular polarity. Interestingly, the cells within the confluent regions of enzyme-treated cultures exhibited increased polymerization of actin, particularly the cells of the uppermost layers of the confluent multilayer or spheroid-forming cultures (Figure 10D-F). These cells exhibited prominent cable formations and accumulation of subcortical polymerized actin lacking any directional preference. In summary, treatment with serine proteases caused a decrease of actin organization within tumor cells at the margins of the wound and a significant increase of multipolar actin microfilaments in cells located in the confluent regions of the monolayers. We hypothesized that the fate of F-actin should be inversely related to the G-actin abundance. This was confirmed in cells situated at the edge of wounds as staining with anti-G-actin antibodies revealed an increased presence of G-actin following protease treatment (Figure 11B). G-actin staining was also consistently more intense following enzyme treatment in cells located within the confluent regions of the cell culture (Figure 11D). Thus, deposits of G-actin increased in all protease-treated cells regardless of whether such cells were released from or confined by cell-cell interactions at the time of protease addition.

Immunochemistry of tight and adherens junctions components. Previously, the induction of cell aggregates by trypsin was thought to be associated with the rapid formation of tight junctions between cells (48). To date, we could not detect specific localization of occludin or ZO-1 antigens by immunohistochemical methods (data not shown). Regardless, the staining with anti-cadherin antibodies revealed a diffuse distribution in control HEK293 cells (Figure 12A). In 3-dimensional structures formed by 24-h treatment with spheroid-forming levels of TR/CH, the cadherin staining became organized between neighboring cells. Experiments in progress suggest that the morphology of spheroids depends on the intensity of the cadherin expression by the cells. We believe that these cadherin-cadherin associations facilitate organization of the aggregated cells into compacted spheroid structures (MCF-7, HEK 293). Cells that are less efficient in cadherin expression, such as highly metastatic B16F10 cells, lack tight associations between aggregated cells and appear as aggregates of more or less rounded cells (see Figure 2f). Finally, cells unable to produce cadherins cannot make spheroids at all (MDA-MB-231; Figure 2i).

Proenzymes vs. active enzymes in cell culture. Selective activation of proenzymes within the tumor environment could account for the apparent antineoplastic selectivity of proenzymes. The effect of proenzymes on the formation of spheroid-like aggregates in confluent B16F10 cells was examined in the presence and absence of serum. In serum-free conditions, the exposure of confluent cells to

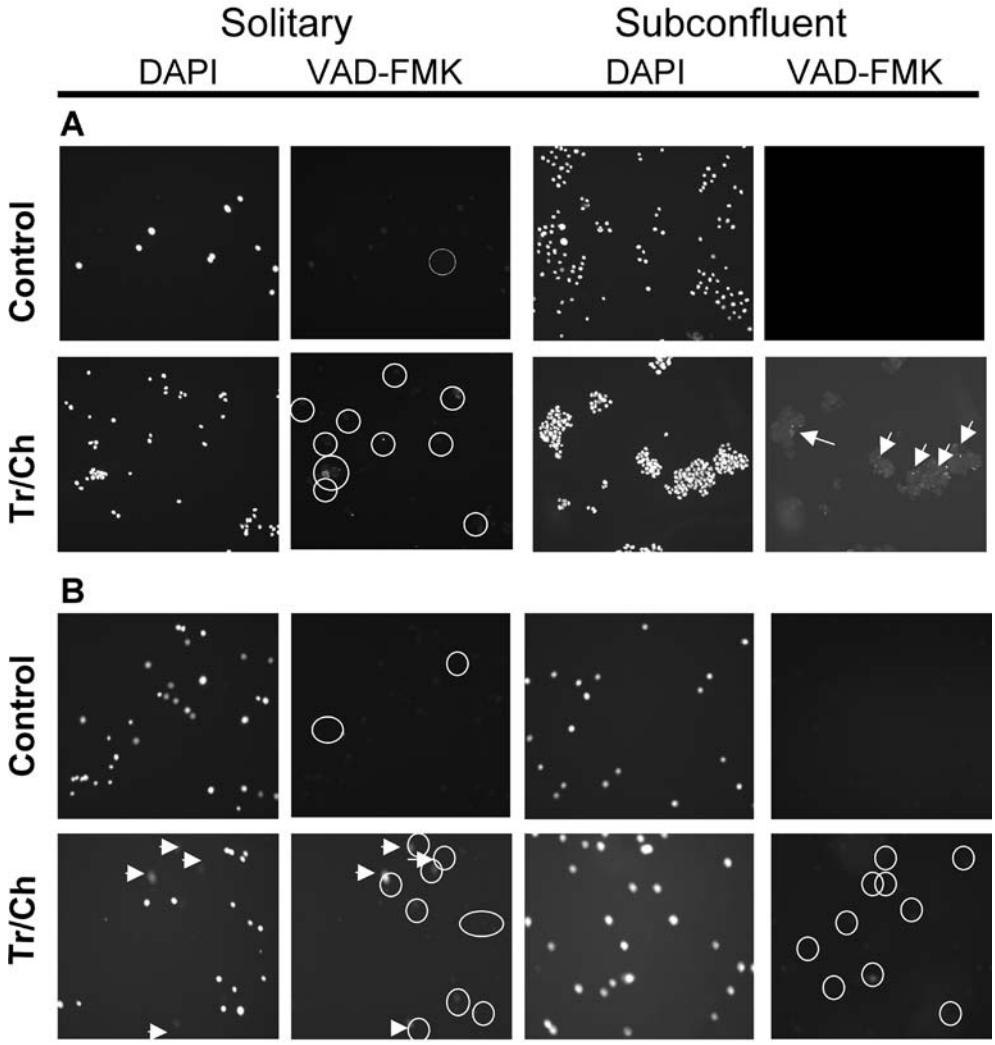

Figure 7. *Induction of apoptosis by TR/CH in tumor sparse and subconfluent cells. Sparse and subconfluent MCF-7 (A) and MDA-MB-231 (B) cells were treated with control or protease-containing (800 ng/ml TR/CH) serum-free media for 5 hours. Following exposure to the enzyme, the cells were detached with EDTA, collected with a rubber policeman and placed on microscope slides for treatment with FITC-VAD-FMK marker, as described in Materials and Methods. The cells were finally mounted in Vectashield containing DAPI. A double exposure of the same cell fields was taken to indicate total cell population (DAPI labeling) and pre-apoptotic cells (VAD-FMK-labeled). As the fluorescence of VAD-FMK-positive cells is lower than that of DAPI label, the positively-labelled cells were placed into the circles in the figures.*

proenzymes led to spheroid formation, although the proenzymes had to be added in higher concentrations to accomplish the same result as active enzymes (see Figure 2). The formation of spheroids was delayed, but not completely eliminated, by increasing the level of serum in culture media. Proenzymes (1600 ng/ml TG/CG) were able to produce cell aggregates even in the presence of 5% serum (results not shown). Interestingly, both enzymes and proenzymes inhibited closure of the wounded monolayers of the highly metastatic MDA-MB-231 cells (Figure 13). In contrast, the accelerated healing of normal cells was accomplished only by enzymes, but not by proenzymes. We conclude that the functional parity of proenzymes and enzymes in spheroid and wound healing assays is based on the selective property of the tumor cells to activate proenzymes. In contrast, normal cells cannot activate serine protease proenzymes and consequently are unable to close the wound in the presence of the TG/CG mixture.

Matrigel invasion assay. B16F10 cells, when placed on the serum-free Matrigel gel in 96-well plates, invaded the matrix and formed small cell aggregates within and at the bottom of the gel (Figure 14A-B). A significant amount of invading cells reached the bottom of the wells. The addition of 200 ng/ml

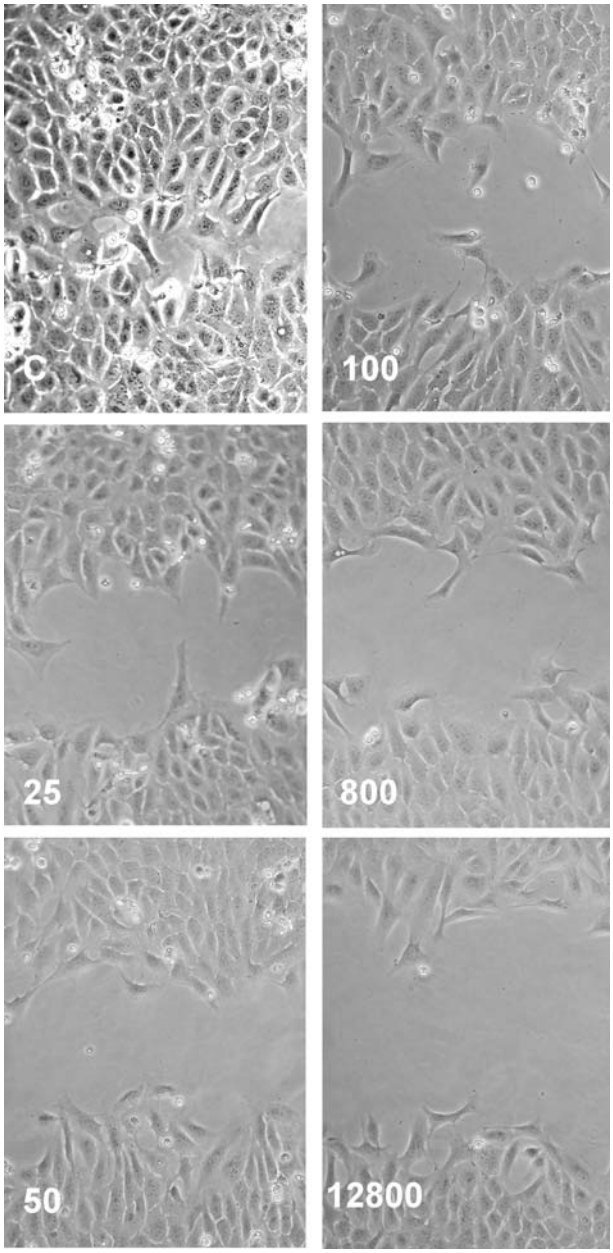

Figure 8. *Proteases prevent migration of neoplastic cells in an in vitro wound healing assay. The confluent cell lawn of U-2OS cells was prepared in 12-well plates. The cells were washed twice with warm serum-free media and wounded during the first washing. Wounded monolayers were incubated with control (C) or TR/CH (ng/ml)-containing media, as indicated in the figure. The progress of the directional migration of cells was recorded 8 hours after wounding.*

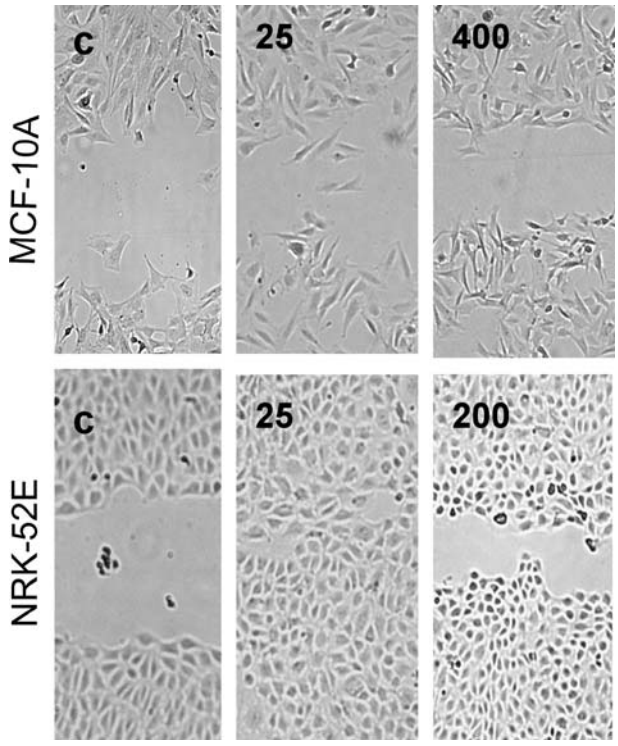

Figure 9. *Low levels of proteases promote migration of normal and immortalized cells. Monolayers of normal epithelial breast cells MCF-10A and normal rat kidney cells NRK-52E were wounded and experiments conducted as described in Figure 8. The wounded monolayers were incubated in control (C) and protease (TR/CH, 25-400 ng/ml)-containing media as indicated in individual images. The migration of cells was recorded after a 12-hour incubation period.*

TG/CG into the gel produced numerous small spheroids on the surface and the formation of large spheroids on the bottom of the wells (Figure 14C-D). It is possible that the aggregates formed originally within the gel, but the continual liquefaction of the Matrigel caused the aggregates to sink to the bottom. Furthermore, the simultaneous addition of tumor cells and proenzymes onto the serum-containing Matrigel resulted in the formation of large spheroids and aggregates on the surface, while effectively preventing tumor cell invasion and/or liquefaction of the gels (in preparation).

Proenzyme therapy of experimental tumors. Methylcholanthrene primary tumors were induced in mice, as described in Materials and Methods and in Trnka *et al*. (42). The tumors, that developed within 37 to 44 days after application of the carcinogen, were randomly distributed into 4 treatment groups. The average size of the tumor at the time of randomization was 10.4 mm^2. The mice were treated by injections of a mixture of amylase (6.68 U) and trypsinogen (750 U) [1 x TG/AM] and the multiples, 10 x TG/AM and 30 x TG/AM. Figure 15 shows that the maximal survival of the control saline-treated mice was less than 67 days. The growth of methylcholantrene-induced tumors was significantly retarded by all treatment modalities; however, there were statistically insignificant differences between individual treatments. The treatment with 30 x TG/AM produced a slim survival rate surpassing 100 days, however, the surviving mice

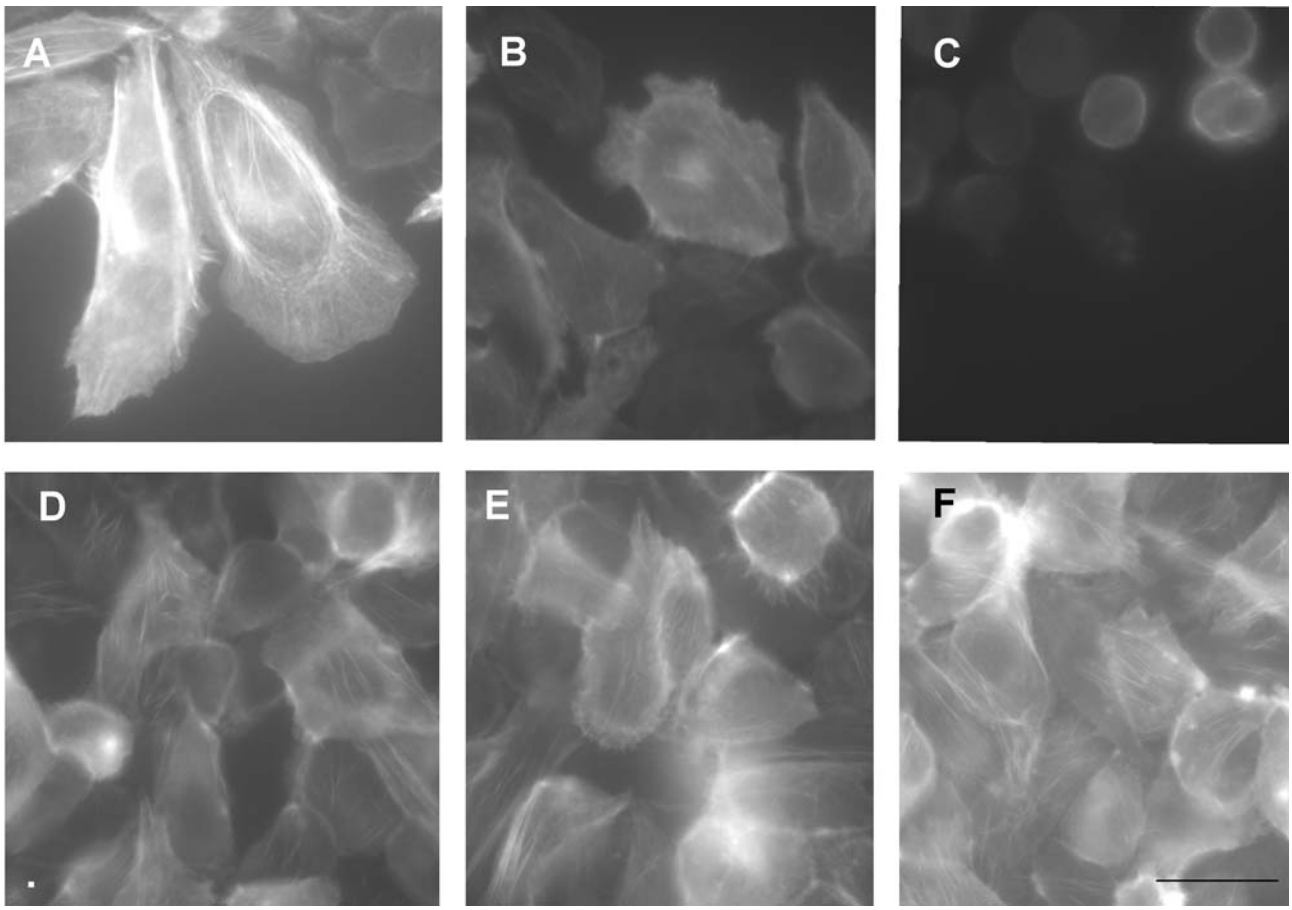

Figure 10. *Reorganization of microfilaments in wounded tumor cell cultures following treatment with proteases. Confluent PC-3 cells were wounded and stained with phalloidin-FITC 12 hours post-wounding. Fluorescence images of cells located at the edge of the closing wound (A-C) and the undisturbed confluent cell layer of the cultures (D-F) are shown: controls (A, D); treatment with 100 ng/ml TR/CH (B, E); treatment with 200 ng/ml TR/CH (C, F). Photographs were obtained with fluorescent microscope under the constant conditions of gain and contrast. The bar in part F indicates 20 μm.*

died shortly afterwards. For better assessment of the antimetastatic potential of TG and AM treatment, we used a B16F10 murine melanoma model of metastasis (49). The cells were injected intradermally into the left flank of the C57Bl6 mice and the tumors were allowed to develop for 10 days. On day 10, the tumors were excised under general anesthesia. Day 10 was selected on the basis of preliminary results indicating a sufficient time to establish metastases and maximum survival time of 42 days. The mice were treated with active agents *via* subcutaneous injections into the flank opposite the tumor every 24 or 48 h, as indicated in Figures 16A and 16B. The enzymes were applied in a final volume of 0.05 to 0.1 ml. Survival of the mice was monitored every day for 100 days. An autopsy was performed on each mouse to determine the extent of the metastasis and, possibly, the cause of death. Operated mice were treated from the day of surgery with either a single agent (TG or AM alone) or a mixture of various strength. Control mice were treated with 0.05 ml injections of saline every 48 h. Amylase alone (0.05 ml of 133.3 U/ml) and trypsinogen alone (0.05 ml of 15,000 U/ml), injected at 48-h intervals, resulted in 20% survival (Figure 16A). Finally, in a direct effort to duplicate John Beard's experiments, a crude bovine pancreatic extract was prepared (42). The extract was adjusted to 1 x concentrations of trypsinogen (750 U) and amylase (6.68 U). The extract, in addition, contained chymotrypsinogen and traces of the active chymotrypsin as determined with the Glu-Ala-Ala-Pro-Phe-Phe-p-nitroanilide substrate. The crude extracts were administered in 0.1 ml aliquots at intervals of 48 h. Remarkably, 60% of mice survived the 100-day test period (Figure 16A).

Although the single agents resulted in a significant therapeutic outcome, the results obtained with the pancreatic extract indicated that the agents provided as a mixture may have surpassed the activity of single agents. The mixture of amylase and trypsinogen, provided once a day, resulted in 40% survival in a 100-day time period

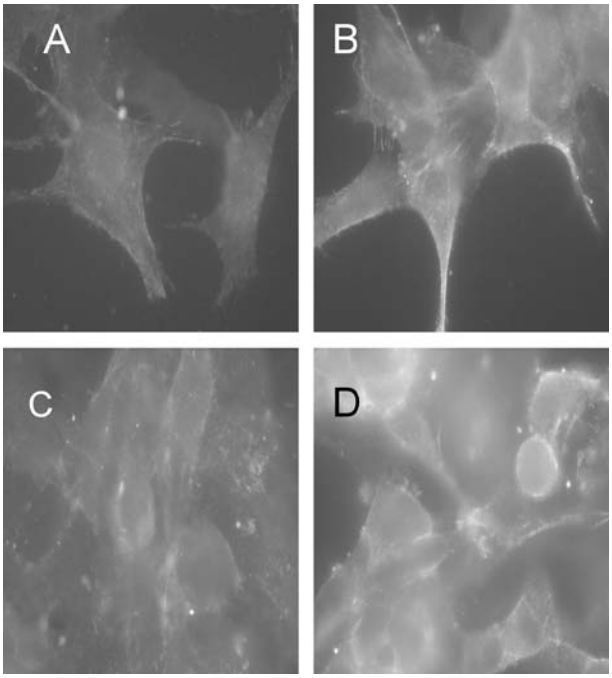

Figure 11. *Accumulation of free actin in TR/CH-treated tumor cells. MG-63 cell monolayers were wounded and cells were treated with TR/CH (100 ng/ml). The cultures were fixed and subjected to immunochemistry 12 hours post-wounding. The localization of soluble actin was accomplished with primary antibodies against G-actin. The images depicting cells at the edge of the wound (A, B) and within the confluent part of the monolayer (C and D) are shown. Untreated controls (A and C) and TR/CH-treated cultures (B and D) were captured under identical gain/contrast conditions.*

(Figure 16B). The same mixture at double enzyme concentrations given every 48 h produced a 60% survival rate in 100 days. When only a half dose of the basic mixture was used every 48 h, the survival rate declined to 20%.

Anti-angiogenic peptides are elevated in proenzyme-treated tumor-bearing mice. Blood levels of angiostatin and endostatin were measured in tumor-free and B16F10 melanoma-carrying mice. Normal and tumor mice were treated with proenzyme (TG/CG/AM) mixtures at the dose levels indicated in the legend of Figure 17. Blood plasma was obtained at various time intervals after the injection of proenzymes and the presence of angiostatic peptides ascertained. As determined by a mouse-specific endostatin immunoassay (Neogen Corp.), high endostatin levels were present in the blood 5 h after injection of the proenzymes. This increase was observed only in the tumor-bearing mice. Some elevation of blood endostatin was still detectable at 8 h post-injection and, thereafter, there was no difference between the treated and untreated mice. In additional studies, we determined that endostatin levels peaked at approximately 3-5 h post *s.c.* injection of proenzymes (in preparation).

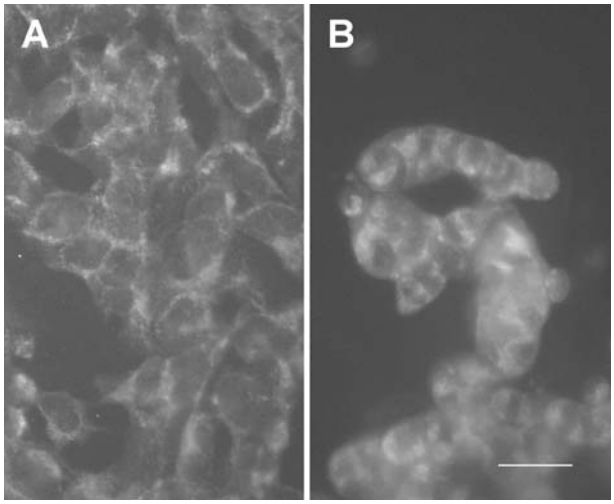

Figure 12. *Cadherin staining of 2- and 3-dimensional cultures of HEK293 cells. HEK293 cells were seeded on glass cover slips and treated with control or TR/CH (800 ng/ml) in DMEM/F12 media for 24 hours. HEK293 cells growing on a glass substrate form compact-type spheroids at lower concentrations in comparison to the polystyrene surfaces (compare with Figure 1). At the end of the incubation, the cells were processed for immunohistochemistry using pan-cadherin antibodies (Santa Cruz). A diffuse, membrane-associated cadherin staining in control (A) cultures is converted into a well organized pattern in compact-type spheroids formed in the presence of the TR/CH (B). The bar indicates 50 μm.*

Angiostatin was determined by means of Western blotting in parallel studies using similar plasma samples. Angiostatin was also detected at 5 h in tumor mice treated with proenzymes, but not in tumor mice without treatment or non-tumor mice with proenzyme treatment.

Discussion

It has long been understood that effective elimination of metastasis would greatly improve the prospects of most tumor patients, if not pave the way for a cure. The concept of the protease-dependent metastatic process (50-52) led to the development of scores of inhibitors against various proteases, particularly the metalloproteinases and serine proteases. To date, only a few such protease inhibitors have shown significant therapeutic potential in the clinical setting (53-55). Efforts, however, continue to prepare new compounds (56), using various inhibitors in combinations (57) or as part of novel therapeutic strategies (58). Thus, our desire to understand John Beard's pioneering explorations into the use of proteases in cancer treatment, as well as our own investigations and results, were often counter intuitive to current research and drug development trends. The results of many well-designed studies indicate the tumor-enhancing activities of endogenous serine proteases. Trypsinogens 1 and 2 are widely expressed

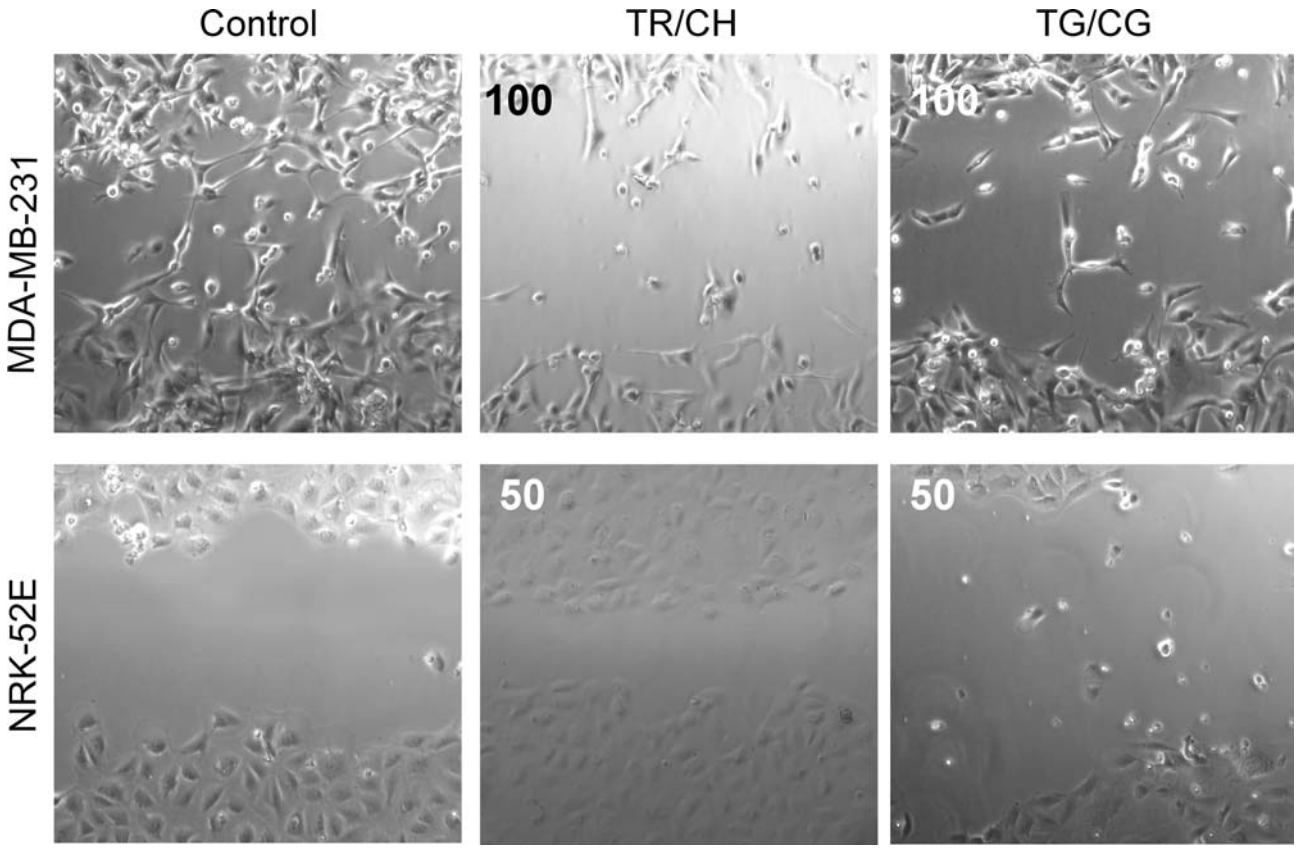

Figure 13. *Tumor but not normal cells exhibit trypsinogen-activating activity. MDA-MB-231 human metastatic breast carcinoma cells and NRK52E normal rat kidney cells were grown to confluency and wounded. Serum-free media were then added along with either TR/CH or trypsinogen/chymotrypsinogen (TG/CG) at concentrations indicated in the Figure. The cultures were photographed at the end of a 12-hour period.*

outside of the pancreas in normal (59, 60) and numerous tumor tissues (61-63). The expression of tumor-associated trypsinogens (TATs) has been, in most tumors, correlated with invasivity (64-65), while in some cases the relationship was deemed obscure (66). Human cancer cell lines transfected with TAT-2 activate numerous pro-metalloproteinases and become invasive in the chick embryo chorioallantoic membrane invasion assay (67, 68). Conversely, inhibition of TAT-2 expression marginally inhibits colorectal carcinoma cell migration in laminin-coated Transwell chambers (69). The activation of trypsinogen proenzymes may occur within the tumor environment by autoactivation (70, 71) or by a membrane-associated cathepsin B (72, 73). Other possible targets of TATs are protease-activated receptor-2 (PAR-2) (74) and prourokinase (75). The latter activation leads to the production of broad-range protease plasmin on the tumor cell surfaces (76). Although tumor progression is mostly associated with overexpression of matrix metalloproteinases (MMPs), the opposite was also noted (46). At least in one tumor system, the expression of trypsin was negatively correlated with the progression of tumors (77) and some tumors, such as hepatomas, lacked expression of TATs altogether (78). As the understanding of metastatic mechanisms is evolving, it may be necessary to re-examine the role of proteases and the utility of their inhibitors in the control of tumor dissemination processes and growth. A successful metastatic cell must be endowed with the following abilities: (i) to detach itself from the primary tumor, (ii) to migrate, (iii) to survive in transit, and (iv) to grow at the future metastatic locus. This communication addresses these processes from the perspective of tumor treatment with exogenous serine (pro)proteases.

Proteases and spheroid formation. The formation of spheroids by protease-treated cells is preceded by distinct structural cell configurations, as described in the Results section (Figure 1). We encountered three different responses to exogenous proteases, specific to a given type of cell: (i) formation of cellular aggregates composed of

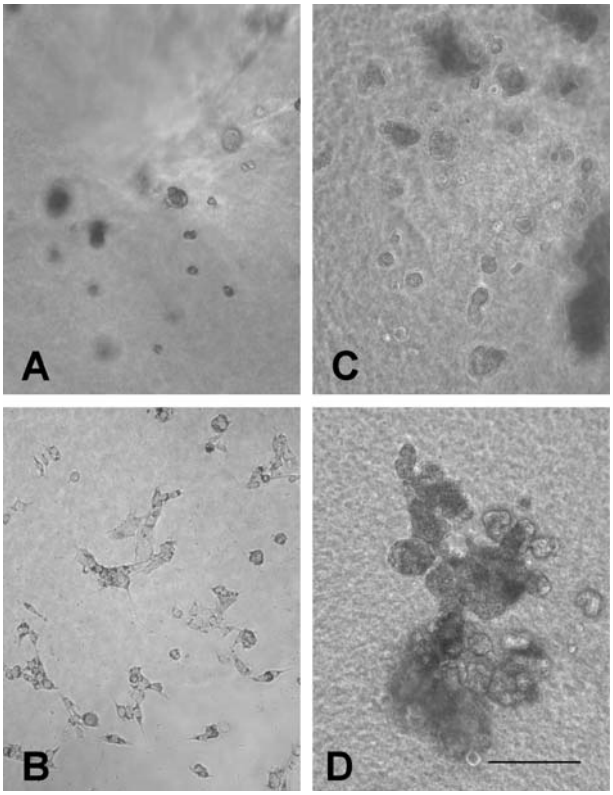

Figure 14. *Invasion of B16F10 cells into Matrigel. Matrigel was prepared in the serum-free media (dilution 1:2) with and without 200 ng/ml trypsinogen/chymotrypsinogen. 0.3 ml of control and Matrigel/proenzyme preparations was added to the individual wells (96-well plate; Nunc Inc). All ingredients were kept at 4˚C prior to mixing and gelling as described in Materials and Methods. B16F10 cells (15,000 cells) were seeded on top of the Matrigel after the gelation at 37˚C was complete (4 hours). The cultures were photographed 2 hours after the addition of cells: control (proenzyme-free) Matrigel (A, B), and the TR/CH containing Matrigel (C, D). Tumor cells in the presence of proenzymes partially dissolved the gels and formed large spheroids within and at the bottom of the wells. Scale bar is 50 μm.*

globular cells, (ii) formation of smooth compact multicellular structures, and (iii) inability to produce aggregates of any kind regardless of the concentrations of the enzymes present. The fate of protease-stressed cells lacking cell-substratum attachments (79) may depend on their ability to engage in cell-cell interactions. The latter may include tight junctions (80) or cadherins (81; Figure 12). The spheroid formation is precluded in sparse cultures void of pre-existing cell-cell contacts and significantly retarded by complete confluency (Figure 3). Finally, we have noted that the propensity to form spheroids depends greatly on the type of culture surfaces. The sequence described in Figure 1 was derived with cells growing on tissue culture-treated polystyrene surfaces. The cells cultured on glass or plastic cover slips require less than half of the protease concentrations to form the same 3-dimensional structures described above for "polystyrene" surfaces. Spheroid formation is also affected by coating the polystyrene surfaces with various extracellular matrix proteins (in preparation). Protease-mediated formation of spheroids encompasses at least three different phases: (i) low concentrations of the exogenous proteases will cause tip-to-tail realignment of neighboring cells; a process underlined by loosening of the cell-substratum attachment as revealed by increased birefringence of the cells (see Figure 1B); (ii) centripetal gathering of neighboring cells based on cell-cell interactions is prompted by intermediate protease concentrations, and, finally, (iii) formation of free-floating loosely attached cell aggregates occurs at still higher enzyme levels and in some cell types the aggregates become compacted into smooth spheroids. The continuous presence of exogenous proteases prevents the detachment of viable cells from the spheroids, a phenomenon that may be relevant to the inhibition of detachment and creation of new metastases *in vivo* (in preparation). The spheroids brought into serum-containing media disintegrate into individual cells and resume 2-dimensional growth and mobility.

Cell aggregation following a 30-sec (500 μg/ml) and 30-min (200 μg/ml) exposure to trypsin has been previously described (82, 83). Subsequent to treatment by trypsin, the aggregation persisted for several hours in the absence of enzyme. Chun (84, 85) showed that exposure of MCF-7 cells to outdated serum results in the production of multicellular spheroids. More importantly, the outdated serum could be replaced with plasmin and/or plasminogen activator and a plasminogen. We were unable to produce a robust formation of spheroids by plasmin at concentrations comparable to those used in this communication (TR/CH at 100 ng/ml; Figure 5). However, sporadic spheroids developed using plasmin concentrations at and above 6.4 μg/ml in MCF-7 cells. It thus appears that the plasmin and plasminogen activator systems do not function as terminal effectors of spheroid formation caused by TR/CH.

Protease treatment, directional movement and cytoskeleton. Results show that proteases prevent tumor cells from repopulating denuded surface following wounding of the confluent cell lawn. The processes of directional movement organized by the lamellipodium (86) are arrested at very low concentrations of TR/CH in all tumor cells, even those that either do not produce spheroids (*e.g.* U2OS; Figures 8 and 2A) or do so only at high concentrations of the proteases (*e.g.* PC-3; Figure 2A). MCF7 cells do not attempt to heal a wound, although they form spheroids at low protease concentrations. This indicates that the inhibition of cell migration is dissociated from the signal leading to the formation of spheroids by tumor cells. In contrast, proteases promote rather than

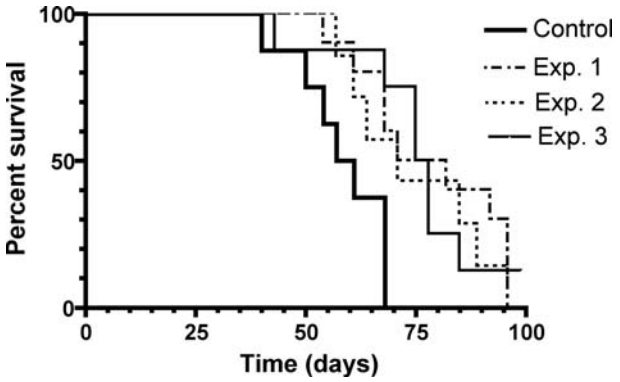

Figure 15. *Survival of mice carrying methylcholanthrene-induced primary tumors treated with trypsinogen (TG) and amylase (AM). The tumors were induced in C57Bl6 mice by repeated application of methylcholanthrene, as described in Materials and Methods. On day 45, the tumor-bearing mice were randomized into treatment groups receiving 750 BAEE trypsin units along with 6.66 amylase units in a total of 0.1 ml volume of saline each day (Exp. 1), 10x multiple of the basic dose (Exp. 2), and 30x multiple of the basic dose (Exp. 3). The injections were delivered s.c. to the opposite flank from that carrying the tumor. The mice were observed daily and the survival noted. The control mice received 0.1 ml of saline only.*

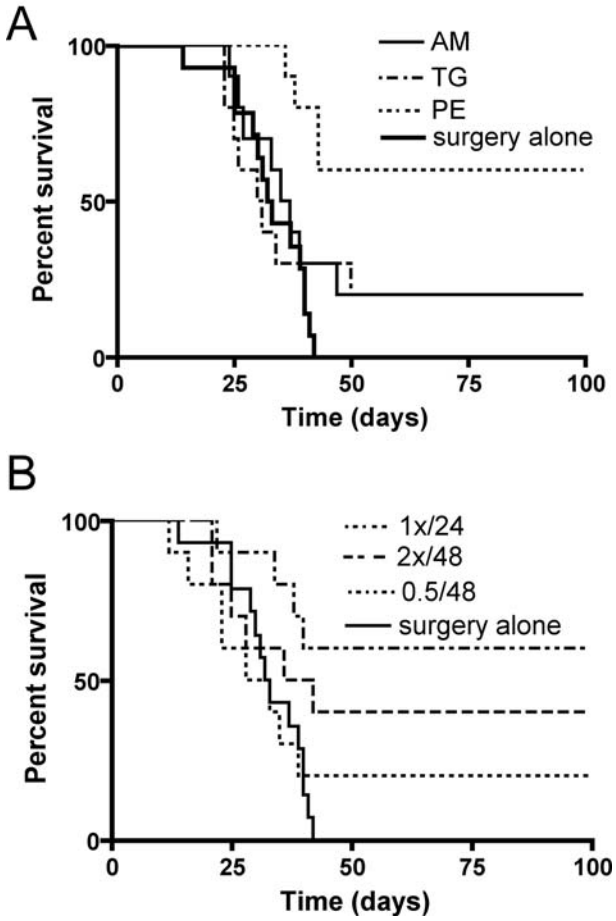

Figure 16. *Survival of B16F10 tumor-bearing mice treated with trypsinogen and amylase. The mice were injected, as described in Materials and Methods, and tumors removed surgically on day 10. Surgery alone extended the life of otherwise untreated mice to a maximum of 42 days. The TG and AM treatments in a total volume of 0.1 ml were delivered subcutaneously immediately after surgery into a flank opposite the tumor. (A) Treatment with the single agents: saline (surgery alone), amylase alone (AM, 6.66 units every 48 hours), trypsinogen (TG, 750 BAEE units every 48 hours), and pancreatic extract (PE; 0.1 ml, every 48 hours). (B) Combination treatments: 6.66 units amylase and 750 units trypsinogen per day (1x/24); 13.25 units amylase and 1500 units trypsinogen in 0.2 ml every second day (2x/48); 3.33 units amylase and 375 units trypsinogen in a volume of 0.05 ml every second day (0.5x/48).*

inhibit the closure of the wounds in monolayers of normal or non-neoplastic cells. This promotion was apparent at concentrations from 50 to 200 ng/ml of TR/CH – the same range of TR/CH that effectively inhibited the closure of wounds in monolayers of tumor cells. It is not clear which molecular entity/mechanism could be responsible for the differential responses by normal and tumor cells to the same proteolytic stimulus. Protease-mediated disorganization of the filamentous actin at the leading edge of the migrating tumor cells (Figure 10) contrasts with the normal appearance of the cytoskeleton in non-transformed cells (results not shown). It is possible that the answer may rest in the differential inactivation of the small GTPases of the Rho family such Rac1 and Cdc42 in tumor and normal cells (87-89).

Spheroid formation and cell-cell communication are survival mechanisms. Polak-Charcon *et al*. (80) suggested a rapid production of tight junctions shortly after treatment with proteases. These authors inferred the formation of tight junctions solely on the basis of morphological features within the cell membrane bilayer as observed by freeze-fracture electron microscopy. We were not able to demonstrate the presence of occludin or ZO-1 in any of the cellular structures induced by exogenous serine proteases. Faff *et al*. (48) observed that the structures of Polak-Charcon *et al*. (80) did not appear to be connected to filaments on the cytoplasmic side. In HEK293 cells that are capable of forming compacted spheroids, we were able to detect cadherin (Figure 12), which in aggregating cells localized along the cell membranes. Preliminary studies indicate that cadherin presence is the reason for the compaction of spheroids. Significantly, the cells lacking cadherins (*e.g.* MDA-MB-231 cells; 90) did not form spheroids (Figure 2A) at any TR/CH concentrations, while cells with intermediary levels of cadherins (*e.g.* B16F10; 91) formed aggregated but not compacted spheroids. The aggregation with or without compaction appears to have an impact on the rate of detachment of cells from formed

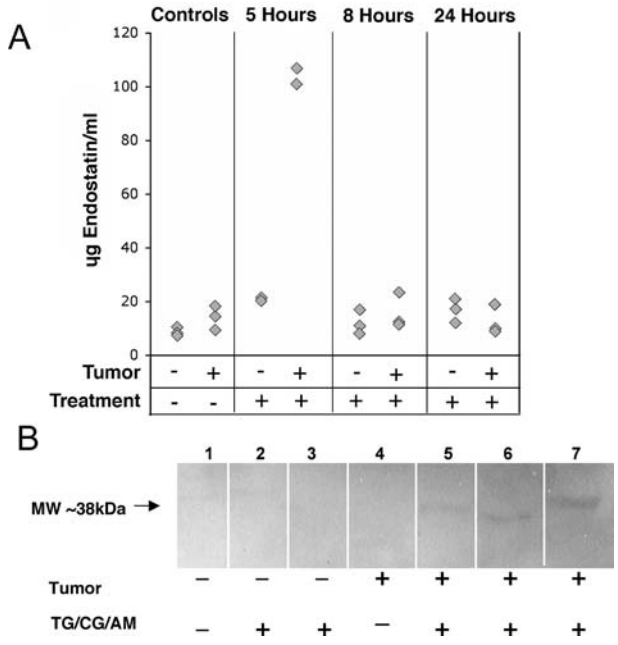

Figure 17. *Generation of angiostatic peptides in plasma of proenzyme-treated tumor-bearing animals. C57Bl6 mice were inoculated s.c. with 10^6 B16F10 cells in a total volume of 0.1 ml of saline. Twelve days following the tumor inoculation, the mice were treated with a mixture of trypsinogen, chymotrypsinogen and amylase (127 µg/kg, 127 µg/kg and 25 µg/kg, respectively). Blood was drawn via cardiac puncture from anesthetized mice into heparin-containing microtubes and plasma separated, as described in Materials and Methods. (A) The endostatin was measured by means of immunoassay (Total Murine Endostatin Immunoassay, Neogen Corp., KY, USA). (B) The level of angiostatin was determined by means of Western blotting using mouse-specific angiostatin antibody, as described in Materials and Methods. Control plasma was from untreated normal mouse (lane 1), tumor-free mice treated with TG/CH/AM (lanes 2 and 3), untreated tumor mouse (lane 4) and three tumor-bearing mice treated with proenzymes (lanes 5-7).*

spheroids, a property that could impact formation of metastases *in vivo*.

The formation of mammalian cell aggregates may be an aspect of the evolution-based survival mechanism that is exhibited by lower organisms such as *Dictyostelium* upon deprivation of food (92) or bacteria under stress (93). Mammalian cells stressed by osmotic pressure (48), or treated with growth factors (94), or by hormonal derivatives (95), also form aggregates. We found that pre-existing cell-cell interactions, or at least the proximity of the cells, are paramount to their ability to form either aggregates or spheroids as a result of protease treatment. Thus, cellular attractiveness and not migration underlies spheroid formation. Cell-cell communication as a determining factor in cell survival was previously postulated by Bates *et al*. (96, 97). In addition, the formation of spheroids is known to be accompanied by acquisition of multicellular resistance (98). Cells maintained in sparse cultures are void of cell-cell interactions. Such cells are rendered susceptible to apoptosis as a result of protease stress. This was shown by labelling with VAD-FITC caspase inhibitor (Figure 7A) as well as by cytofluorimetry based on the uptake of 7-aminoactinomycin D (results not shown). Cultures that were at mid-confluence were able to sequester most of the cells into spheroids upon addition of proteases, leading to low overall apoptosis. Nevertheless, the spheroids contained a variable number of cells carrying an apoptotic marker. It is possible that rapidly coalescing 2- and 3-dimensional pre-spheroid structures sweep outlying singular pre-apoptotic cells into spheroids. Conversely, tumor cells, which are incapable of cell-cell communication, are subject to almost complete elimination by proteases. Thus, both sparse and subconfluent MDA-MB-231 cells exhibit a high level of apoptosis when treated with proteases (Figure 7B). We believe that this differential behavior is based on the presence of cadherins in MCF-7 cells and their absence in MDA-MB-231 cells.

Relationship of protease-induced changes in vitro to survival in vivo. The formed spheroids and clumps consisted mostly of living cells exhibiting low proliferative and metabolic indices (Figure 6). Such structures may resemble tumor clumps freely floating in the blood (99), lymph streams (100), migrating through the extracellular matrix of the connective tissues (101, 102), or vasculature-lodged small groups of cells (103). Different tumor cells may have varied capacities to survive in a semi-adherent or non-adherent state. The process of cell death may be delayed by maintenance of the multicellular structure (104). We have observed that protease-derived spheroids maintained in serum-free media (*e.g.* VP-SFM, Invitrogen) require intermittent exposure to proteases to maintain the spheroid-like morphology and intermittent exposure to serum to remain viable (unpublished results). This is consistent with the preliminary results of proenzyme therapy of experimental tumors in which the growth of primary tumors is only slowed (but not eliminated) and cessation of therapy may lead to a renewed and vigorous growth (in preparation). The antitumor effect of proenzyme therapy may thus lie in the specificity of the proenzyme attack on metastatic cells, particularly single cells in transit (102). Such cells are in the process of establishing foci (105) and, thus, are not yet protected by multicellular resistance against external or local (*e.g.* immune) factors and anoikis (106).

Inherent to the induction of cell-cell communication is the resistance of cells to disperse from the formed spheroid. Preliminary data show that, whereas picomolar concentrations of enzymes are needed to form spheroids, subpicomolar concentrations are sufficient to maintain 3-dimenional structures. In addition, we are finding that

most of the detached cells are nonviable or pre-apoptotic. Should the same processes occur *in vivo*, the metastatic spread would be curtailed and the proenzyme treatment would be more efficient against metastatic tumors. To test this hypothesis, studies were conducted on two experimental tumor systems: methylcholantrene-induced tumors and a B16F10 melanoma. While the former tumor is not known to be avidly metastatic (107), the B16F10 melanoma was selected for metastasis dissemination (49). As predicted, parenteral proenzyme therapy of B16F10 melanoma-carrying mice resulted in superior survival results in comparison to the treatment of methylcholanthrene-induced tumors (Figures 15-16). Rectal administration of milligram levels of active protease mixtures (30, 108-110) resulted in high survival rates of mice carrying Lewis lung carcinoma (108) and B16 melanoma (109). It is not known whether the achieved high survival rates are due to the route of administration, dosage or the nature of the active components. Preliminary results directly comparing parenteral administration of active enzymes and proenzymes indicate the substantially lower effectivity of the former (in preparation).

Proenzyme treatment is tumor-specific and angiostatic. We have shown that protease-formed spheroids switch to a dormant state characterized by low proliferative and metabolic activities. *In vitro* these spheroids disintegrate into individual living cells which resume normal 2-dimensional growth within 12 h after removal of the proteases from the media. The extension of this observation into an *in vivo* situation would indicate formation of dormant foci, which could reactivate soon after the protease pressure is abated. From the experience of Beard (111, 112), his contemporaries (see Table I; 113), Trnka *et al*. (42) and Novak and Trnka (114), it appears that reactivation of aggregated metastatic foci either does not occur or the foci have disappeared. Permanent suppression of metastatic spread appears to be dependent on intermittent proenzyme treatment over extended time. The length of the treatment has been recommended to last at least several months (15). In an ongoing human study, seventeen terminal patients were treated with proenzymes of which six patients, suffering from as many different neoplasms, responded positively with essentially complications-free life extension from 0.7 to 9.5 years, as previously reported (114). The finding that only tumor-bearing mice treated with proenzymes had elevated levels of endostatin and angiostatin (Figure 17) indicates that the proenzyme treatment includes an angiostatic component. Elastase (115) and cathepsin L (116) were identified as possible activators of endostatin. Plasmin (117, 118) and macrophage-derived metalloelastase (119) can convert plasminogen into angiostatin. As most of the proteases are synthesized and excreted as proenzymes, their activation could be a key step in the generation of anti-angiogenic compounds. The inactive proform of elastase can be activated by trypsin (120). A direct effect of TR/CH on collagen type XVIII and plasminogen may also generate the endostatin-like and angiostatin-like peptides, respectively (121, 122). The generation of anti-angiostatic peptides exclusively in tumor-bearing animals (Figure 17) complements the *in vitro* activation of trypsinogen/chymotrypsinogen by tumor but not normal cells (Figure 13).

Proenzymes, Beard's discovery and unresolved questions. The concentrations of proenzymes and enzymes used in cell experiments, experimental tumors and patients were derived from published data (15). Beard did not have purified proenzymes/enzymes at his disposal and measured the amount of pancreatic extracts in Robertsonian units. Although the latter are long-forgotten formulae (123), it was possible to convert the dosages Beard used nearly one hundred years ago into weight or BAEE units. According to these calculations, the amount of trypsinogen and amylase was determined for each mouse injection: 31.25 BAEE units TG/g mouse and 27 U amylase/g mouse. In the current murine trials, we found that the most effective daily dosages of a combination of trypsinogen/chymotrypsinogen/amylase are somewhat higher when injected intramuscularly or subcutaneously (in preparation). Since *i.m.* or *s.c.* injections absorb at slow rates, the actual blood concentration of the proenzymes could be a fraction of the initial injection. Several seemingly insurmountable problems stand in the way of quantitative analysis and the pharmacological disposition of the active compounds. Equally difficult will be the comprehension of some of the mechanisms involved. First, the large molecular weight of the tested substances challenges our present understanding of transepithelial transport. A second set of problems stems from the inherent biological liability of the large molecules to clearance by a variety of scavenger receptor systems in the liver and other tissues (124), *via* kidney function (125), recognition by an immune system (126, 127), and *via* chemical and proteolytic destruction (128). It should be noted that no serious immune reactions or any other toxicity have ever been reported in response to administration of pancreatic enzymes or proenzymes in humans or rodents (15, 42, 111, 114). As documented in this communication, the low general toxicity and high tumor/normal tissue pharmacological efficiency may rest on the finding of selective activation of proenzymes by tumor cells. This would assure avoidance of serine protease-type inhibitors that activated enzymes would instantly encounter if administered parenterally. The insistence by Beard (15) on the use of "fresh" pancreatic extracts is testimony of his genius long before the biochemistry of the pancreatic proenzymes had been described. Similarly, daily application of proenzymes to animals or patients has

never elicited a phenomenon of specific or pleiotropic resistance (129). Future research should concern all of the unresolved problems suggested in this work as well as repeating Beard's findings in controlled clinical trials.

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