Effect of Inhibitors from Adult Connective Tissue on Growth of a Series of Human Tumors in vitro

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SUMMARY

The extraction of growth inhibitors from normal adult connective tissue (aorta, tendon, and muscle) by mild trypsin digestion is described. More than 100 growth inhibitory preparations, including crude tissue extracts, fractions, and subfractions were tested for their effects on growth in primary tissue culture of 25 biopsies of human tumors. The growth of many tumors was reduced 75–100% when incubated in these solutions before being cultured. The preparations were particularly effective against sarcomas, but inhibited the growth of other tumors as well (melanomas, hemangiopericytomas, a xanthoma, and a thymoma); the supporting mesenchymal cells of other tumors were also inhibited. These results appear to support the thesis that a balance of stimulatory and inhibitory substances controls growth normally.

Histochemical studies indicated that these agents are normal components of the intercellular ground substance. They are nondialyzable macromolecules, highly active biologically, since very small amounts (0.05 mg/ml or less) clearly showed the effects described. Nitrogen values indicated at least 50% protein. No deoxyribonucleic acid (DNA) was present but there was approximately 10% ribonucleic acid (RNA). Sensitive chemical tests failed to reveal significant amounts of hexoses or hexosamines. It appears likely that these factors may be part of complexes associated with proteins such as mucopoly saccharide-nucleic acid-protein complexes.

There is accumulating evidence that biologically active factors (growth stimulating and growth inhibiting substances) present in normal adult tissue form a growth control mechanism which is responsible for the dormancy of normal adult tissue. An upset of this balance, or the absence of the inhibiting factor, may result in the unrestrained growth of cancer. The thesis that neoplasia may represent a disturbance of equilibrium between growth stimulating and growth inhibiting substances was proposed by Murphy (7). Paul (14) in a recent review of the cancer cell in vitro, stated that "Cancer is clearly the result of a defect of the homeostatic mechanisms which maintain the balance among cells in the organism." The isolation and characterization of specific growth factors from normal adult mice by Levi-Montalcini and Cohen (2, 6), as well as the extraction from normal thymus and other tissues of "promine" and "retine" by Szent-Gyorgyi et al. (5, 19–21) has renewed interest in this hypothesis.

Simms and Stillman (17, 18) described the removal of a "tissue inhibitor" present in adult tissue and in tissue cultures of adult tissue, by enzymatic digestion. Later Simms and Parshley (16) observed that either growth stimulators or inhibitors were produced by enzymatic digestion of adult chicken aorta depending upon the conditions of digestion. About 3 years ago I (10) reported the separation of similar factors from other adult mesenchymal tissue, i.e., tendon and skeletal muscle. Growth stimulating factors are more readily soluble; they are released by a lower concentration of the enzyme and are more stable. The growth inhibitors, constituents of normal adult tissue, were tested for their effect on growth in vitro of adult and embryonic connective tissue cells from aorta and heart. They restrained the growth of fibroblasts in tissue culture by 75–100%.

Since that time we have tested the effect of these inhibitors on the growth in vitro of a series of human tumors. The extracts have completely or partially inhibited the growth of sarcoma cells as well as the cells of other types of human tumors. They appear to be particularly effective against cells of mesenchymal origin. A preliminary report of this work was presented at the 13th Annual Meeting of The Tissue Culture Association (11). These inhibitors have also prevented the growth in vitro or completely destroyed the cells of several strains of highly malignant human tumors (13). Active fractions have been subjected to column chromatography, and the resultant subfractions tested for their effect on the growth of normal and malignant human cells, and against tumors in mice and rats.
Preparation and fractionation of growth inhibitory extracts.

Crude extracts were prepared from chicken and dog aorta, beef tendon, and chicken skeletal muscle by conditions of proteolytic digestion found to be optimal for the extraction of inhibitors. Best results were obtained with prolonged digestion of the tissue at low temperatures. The tissue was handled steriley throughout.

In a typical experiment (Chart 1) an inhibitory extract, 13-PM, was prepared from adult chicken skeletal muscle. Approximately 13.6 gm of muscle were chopped finely and combined with 20 ml of 0.1 % Difco trypsin 1:250 in Moscona solution (containing per liter distilled water: 8 gm NaCl, 0.2 gm KCl, 1 gm NaHCO₃, 0.005 gm NaH₂PO₄, 2 gm glucose, 0.005 gm phenol red). The pH was adjusted to 7.2 and the extract was kept at 4°C. for 24 hr. After 3 hr. the pH, which gradually dropped below 7.0, was readjusted to 7.2 with 0.5 M NaOH. The extract was centrifuged for 1 hr. at 3,000 r.p.m. and the supernatant fluid removed. This preparation had inhibitory activity as shown in Table 1 and Chart 4. To half of this extract at pH 7.4 10 ml of absolute ethyl alcohol were added. After 24 hr. at 4°C the pH had dropped to below 7.0 with the formation of a white precipitate. The material was centrifuged again and the supernatant removed. The precipitate weighed 1.4 gm, corresponding to 6.8 gm muscle (wet weight); it was lyophilized and later brought into solution in a volume of Moscona solution equal to the original 10 ml (13-PM-A). To 10 ml of the supernatant was added 0.2 ml of 5 % CaCl₂ and 1 drop of 0.5 M NaOH to bring the pH to 7.6. This was left at 4°C. for 24 hr., centrifuged, the supernatant removed and discarded, and the precipitate weighed. It weighed 0.9 gm, corresponding to 3.4 gm fresh tissue. This precipitate was dried and later brought into solution in 5 ml Moscona solution and tested (13-PM-Ca). This material also had inhibitory activity against tumor cells (Table 1 and Chart 4).

Extract 15-BT was prepared in a similar way using beef tendon instead of muscle as a starting material (Chart 2). Approximately 10 gm of tendon were chopped finely and combined with 20 ml of 0.2 % Difco trypsin 1:250 in Moscona solution. The pH was adjusted to 7.2 and the extract kept at 4°C. for 24 hr. When this had clotted, a supernatant medium (25 % human placental serum, 25 % serum ultrafiltrate in BSS) was added and the pH was adjusted to 7.4. The flasks were incubated at 37°C. for 2–4 days. After this treatment the 4 strips of fresh tumor tissue (about 1.5 X 2.0 mm) were placed in each of a series of small test tubes with 1.5 ml of test solution, usually containing 10 % (but sometimes 33 %) human placental serum. There were 2 control tubes for each experiment containing the same percentage of serum in balanced salt solution (BSS). The pH was adjusted to 7.4 with 5 % CO₂ in air and the tubes were incubated at 37°C. for 2–4 days. After this treatment the 4 strips of tissue from each tube were cut into 32 equal cubes and planted in even rows in each of two 3.5-cm Carrel flasks, in a thin layer of dilute chicken plasma.

Tests for growth inhibition in primary tissue cultures.—

The method used for testing has been described in detail elsewhere (12, 17). It consists of incubating the tissue in the extracts, also containing serum, before cultivation in a highly stimulating growth medium. During incubation of the tissue, trypsin, if still present in these extracts, is inactivated by the serum (which contains trypsin inhibitor). If under these circumstances there is no growth of tissue treated with inhibitor, as compared to a luxurious growth of control tissue, it is evident that the extracts contain a component inhibitory to the growth of tumor cells. In detail, 4 strips of fresh tumor tissue (about 1.5 X 2.0 mm) were placed in each of a series of small test tubes with 1.5 ml of test solution, usually containing 10 % (but sometimes 33 %) human placental serum. There were 2 control tubes for each experiment containing the same percentage of serum in balanced salt solution (BSS). The pH was adjusted to 7.4 with 5 % CO₂ in air and the tubes were incubated at 37°C. for 2–4 days. After this treatment the 4 strips of tissue from each tube were cut into 32 equal cubes and planted in even rows in each of two 3.5-cm Carrel flasks, in a thin layer of dilute chicken plasma. When this had clotted, a supernatant medium (25 % human placental serum, 25 % serum ultrafiltrate in BSS) was added and the pH was adjusted to 7.4. The flasks...
**TABLE 1**

**INHIBITION OF GROWTH IN VITRO OF CELLS FROM HUMAN TUMORS BY GROWTH INHIBITORY EXTRACTS PREPARED FROM NORMAL ADULT CHICKEN AORTA AND SKELETAL MUSCLE AND BEEF TENDON (% INHIBITION AS COMPARED TO CONTROLS)**

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* The abbreviations used are: TA, thoracic aorta; PM, pectoral muscle; BT, beef tendon; Ca, Ca precipitate; A, alcohol precipitate; D, dialyzed.

were kept at 37°C. and observed daily (Fig. 1). The amount of growth, as evidenced by the increase in surface area of the outgrowth, was estimated for each colony by an arbitrary rating (0—3) (Fig. 2). The sum of the ratings of the 16 colonies was taken as the growth value for each flask. The value for each test is the mean of 2 duplicate flasks (i.e., representing 32 explants). The average growth value of the controls is taken as 100% and the extent of growth inhibition in Table 1 is expressed in relation to this.

**Histochemical and chemical tests to characterize the active agent.**—Sections of untreated aorta, tendon, and muscle tissue were compared with sections of the same tissue after trypsin digestion to determine the nature of the material extracted. The following histologic procedures were carried out: hematoxylin and eosin, Giemsa, fast green, periodic acid Schiff followed by Lillie's allochrome technique, Mallory's phosphotungstic acid (PTA) stain, Alcian blue, thionin and HgCl₂, Azure A, Sudan B following chromation, and luxol fast blue. Kjeldahl nitrogen deoxyribonucleic acid (DNA) ribonucleic acid (RNA), hexose, and hexosamine determinations were made.

**RESULTS**

More than 100 crude extracts and fractions were screened in primary tissue culture for any inhibitory effect on normal connective tissue fibroblasts from adult chicken aorta (Table 1, Figs. 3—5). The most effective extracts were tested on a series of human tumors. Of the 25 tumors, 2 failed to grow due to storage for several days before culture in saline containing antibiotics. The others grew at varying rates. The inhibitors restrained the
growth of different tumors 75–100 % (Charts 3–7). The results obtained against 14 tumors with 16 preparations are summarized in Table 1. The effect of 1.5 ml test solution containing 0.5–0.9 mg N₂/ml is reported. Cells from tumors of mesenchymal origin and fibroblasts in other types of tumors were affected most. In many instances normal fibroblasts from adult chicken aorta were less affected. The growth of sarcoma cells was inhibited 75–100 %, in many instances 100 % (Charts 4 and 6, and Figs. 11 and 12). Fibroblastic elements of the carcinomas were also inhibited (Table 1, Figs. 7 and 8). The epithelial elements of 1 of 3 breast carcinomas appeared to be affected. The epithelial cells of a thymoma which grew luxuriantly (Fig. 6) were inhibited by 15-BT, as were the fibroblasts (Chart 3). Several extracts inhibited the growth of melanoblasts 100 % (Table 1 and Chart 5). Cells from 2 types of melanoma are shown (Figs. 9 and 10). The pericytes from a hemangiopericytoma were also
inhibited (Chart 7 and Figs. 13 and 14). The histiocytes of a xanthoma were inhibited 75% (Table 1). In 7 experiments the injection of growth inhibitory preparations gave 40–100% protection against animal tumors, with no deaths in the experimental series. These results correlate well with the tissue culture results; they infer a biologic and not a toxic action on the part of the agent. These experiments will be reported in detail at a later date.

Some differences between preparations were observed consistently. Crude extracts of aorta and muscle remained stable at 4°C. for several months, while tendon extracts lost activity earlier. Sarcomas were completely inhibited by extracts 6–12 months old which had lost their effect on normal cells. Several extracts were found to stimulate growth of normal cells with loss of inhibitory effect (Table 2), indicating the presence in the extracts of a complex of inhibitors with more stable stimulators. The stimulators can be separated from the inhibitors by DEAE cellulose column fractionation (15). The agents resisted heating to 58°C. but were partially destroyed by heating to 100°C. (Chart 4). Activity remained after dialysis through Visking cellophane tubing (Chart 7). Active agents were precipitated from aorta and muscle, but not from tendon, by ethanol. They were precipitated from all 3 tissues by CaCl₂ (Table 1 and Chart 4). These precipitates were more stable than the original extracts. If growth was not completely inhibited, the effect of the inhibitors in tissue culture was to increase the lag period and decrease the rate of migration and cell division, as measured by the extent of the cell outgrowth from the explant. Cultures of normal fibroblasts showed loss of cohesiveness and increased fat deposition (Figs. 3–5). Mitosis was observed in some cultures, although the cells appeared shriveled and degenerate (Fig. 12). Increased lipid in fibroblast cultures was demonstrated with Sudan Black B, and loss of production of metachromatic ground substance elements with Toluidine Blue O.

Sections of tissue from which the inhibitors had been extracted were compared with sections of untreated tissue by various histochemical procedures. With hematoxylin and Giemsa there was an apparent loss of intercellular material from aorta, tendon, and muscle septa. The nuclei were gone from tendon sections but were present, though pyknotic, in sections of aorta and muscle. There was a considerable loss of protein from tendon and muscle, some loss from aorta (fast green), and loss of periodic acid Schiff positive elements from all 3. Counterstaining with Lille's allochrome technic, as well as staining with Mallory's PTA stain, indicated that collagen, reticulin, and muscle were not involved, while various technics which demonstrate the components of the ground substance mucopolysaccharides, sulfonated mucopolysaccharides, and hyaluronate (Alcian blue, thionin and HgCl₂, and Azure A) showed loss of these substances. These results lead me to believe that these agents are normal components of the intercellular ground substance. Their chemical nature has not yet been determined and it is quite possible that several distinct inhibitory compounds are involved. Nitrogen values indicated at least 50% protein. There was no DNA present, but approximately 10% RNA. However, sensitive chemical tests failed to reveal significant amounts of hexoses or hexosamines (1–2%).

DISCUSSION

Tumors are known to lack many chemical constituents characteristic of normal tissue (4). In 1936, Murphy (7) presented the hypothesis that intracellular changes in carcinogenesis were "due to a break in the equilibrium between inhibitory substances of growth and excitatory
substances of growth. In a long series of experiments
(8, 9) he and his co-workers extracted substances from
slow growing Rous sarcoma which inhibited the growth
from similar tumors when injected into chickens and rats.
They isolated similar inhibitors from normal skin and
placenta. In the Rous sarcoma the inhibitory agent was
associated with the active causative principle of the
tumor. The active principle gave a strong Feulgen
reaction for nucleoprotein, while the inhibitor gave a weak
reaction. The inhibitory precipitate stained predomin-
antly blue with Mallory, indicating a component of
connective tissue associated with the inhibitor activity.
It was effective against mouse sarcoma, but not against
tumor. The active inhibitory principle was low in protein and probably a
hydrated carbohydrate. A mucoprotein with properties similar to
those of chondroitin sulfate could be precipitated out and was
not associated with the active principle.

Reports of experiments in which the tissue culture
father has been used to evaluate the effect on growth
support the view that normally an equilibrium between
inhibitors and stimulators regulates growth. Simms and
Stillman (17, 18) isolated a growth inhibitor from adult
chicken aorta by mild tryptic digestion. This extract
inhibited the growth of normal adult chicken fibroblasts.
Cultures which had ceased to grow showed a renewed
spurt of growth following tryptic digestion. They
considered this to be a result of the removal of an inhi-
bitor from the intercellular material to the digestive
fluid. This fluid inhibited growth when added to other
cultures. Brues et al. (1) reported that aqueous and alco-
hol extracts of adult liver inhibited growth in vitro
of fibroblasts from chick embryo heart, and also from mouse
Sarcoma 180. The factor which Brues considered
analogous to Simms’ factor was not present in embryonic
tissue and appeared to be specific for mesenchymal tissue.
Fogh (3) recently reported inhibition in tissue culture of
those of chondroitin sulfate could be precipitated out and was
not associated with the active principle.

There is considerable interest at the present time in the
2 growth stimulating and inhibiting substances, promine
and retine, isolated by Szent-Gyorgyi et al. (5, 19–21).
These materials, extracted originally from the thymus
gland, and recently from aorta, muscle, tendon, and
urine, are considered by the authors to constitute a normal
growth control mechanism. An upset of the balance
of these factors may result in unrestrained growth. Retine
cause complete regression of tumors in mice. Szent-
Gyorgyi considers the inhibitor to be a highly active
biologic factor or factors of small molecular weight. In
Polysaccharides in Biology, M. J. Shear (15) described the
isolation of another highly active inhibitory agent from
bacteria and plant material. He and his associates found
that this material, when injected into animals with
sarcoma, was capable of destroying the tumor. The
effective agents were expected to be protein in nature, but
were found to be polysaccharides.

My results appear to support the earlier findings that
complexes of growth factors in normal tissue growth
control. There must be an infinite variety of these
materials in the different tissues in view of the many
sources listed in the literature and the variation in physio-
logic and chemical properties (1, 2, 8, 9, 15, 20–22). In
my experiments I have dealt essentially with factors
present in normal connective tissue. The individual
variation in response in these experiments indicates that
there are many factors in this category. It appears likely
that these factors, macromolecules which are highly active
biologically, are part of complexes associated with proteins
such as mucopolysaccharide - nucleic acid - protein com-
plexes.

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10. PARSHLEY, M. S. Isolation of a Growth Inhibitor from Adult

11. PARSHLEY, M. S. Effect of Growth Inhibitors from Adult

![Figure 1](image1.png)

**FIG. 1.—Tissue explants under cultivation, as described in
text, in 3.5-mm Carrel flask (12).**

![Figure 2](image2.png)

**FIG. 2.—Amount of growth corresponding to the indicated
ratings. 0.5, 10–30 cells, frame of 30+ cells, about 90 new cells,
½ diameter explant; 5, abundant growth extending 0.8 mm from
explant, equal to diameter explant (12, 17).**

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FIGS. 3-5.—Sister cultures, 5 days old, of adult chicken aorta fibroblasts. Fig. 3 is a control. Fig. 4 shows culture incubated in a Ca precipitate of muscle extract (18-PM-Ca) before planting. There is sparse growth of stringy cells with large fat granules (g). Fig. 5 shows culture incubated in the same extract heated to 100°C.; the inhibitory activity has been partially destroyed without affecting deposition of lipid. Harris hematoxylin, X 100.

Fig. 6.—Control culture, 10 days old, of epithelium of human thymoma. Lymphocytes disappear during 1st few days, leaving epithelium and some fibroblasts. Azure, X 100.
FIGS. 7 AND 8.—Human carcinoma of the breast. Sister cultures, 7 days old. Fig. 7 is a control; fibroblasts (f) have greatly overgrown the pigmented epithelium (e). Fig. 8 shows culture incubated in a beef tendon extract (34-BT) before planting; fibroblasts are sparse and shriveled; epithelium is more conspicuous. Harris hematoxylin, X 100.

FIGS. 9 AND 10.—Cultures of 2 different human melanomas. Fig. 9 shows a 14-day-old culture of a heavily pigmented melanoma of the leg; both melanoblasts (m) and fibroblasts (f) contain pigment (pf). Harris hematoxylin, X 100. Fig. 10 shows a 7-day-old culture of a nonpigmented melanoma with metastasis to lymph node; almost no pigment is visible in cells. Fontana for melanin, X 100.
FIGS. 11 AND 12.—Human sarcoma of chest wall. Sister cultures, 7 days old. Fig. 11 is a control. Fig. 12 shows culture incubated in a dialyzed extract of beef tendon (25-BT-D) before planting. Mitosis (m) is occurring, although outgrowth is sparse and shriveled. Harris hematoxylin, X 100.

FIGS. 13 AND 14.—Human hemangiopericytoma. Sister cultures, 4 days old. Fig. 13 is a control. Fig. 14 shows culture incubated in an extract of beef tendon (25-BT) before planting. There are characteristic feathery processes of pericytes (p). Harris hematoxylin, X 100.


Effect of Inhibitors from Adult Connective Tissue on Growth of a Series of Human Tumors *in Vitro*

Mary Stearns Parshley


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