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Melanoma Growth Stimulatory Activity: Isolation From Human Melanoma Tumors and Characterization of Tissue Distribution

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Melanoma growth stimulatory activity (MGSA) is an acid and heat stable, autostimulatory growth factor which was first isolated from culture medium conditioned by the Hs294T human melanoma cell line. In this report, we describe the purification of MGSA from acid ethanol extracts of Hs294T tumors grown in nude mice using a series of Bio-Gel P30, reverse phase-high performance liquid chromatography and heparin-sepharose steps. This modified procedure provides a 10-fold improved yield of MGSA over previously published procedures. Purified MGSA-stimulated melanoma cell growth in both ^{3}H-thymidine and cell number assays over a concentration range of 0.06 to 6 ng/ml. The MGSA bioactivity was primarily associated with fractions which exhibited molecular weights of 16 and 13–14 Kd based upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-1), transforming growth factor-beta (TGF_β), and epidermal growth factor (EGF) in combination with TGF_β did not stimulate ^{3}H-thymidine incorporation in Hs294T cells under the conditions used for MGSA bioassay. Monoclonal antibody to MGSA was used to screen melanoma and benign nevus cultures as well as fixed sectioned tissue for MGSA. The majority of the melanoma cultures were MGSA positive, while most nevus cultures were MGSA negative. However, when fixed sectioned tissue was screened for MGSA immunoreactivity, melanoma tissue was MGSA positive and three-fourths of the benign nevi were MGSA positive. In addition, epidermal keratinocytes and several tissues exhibiting proliferative disorders contained immunoreactive MGSA. These data suggest that MGSA may be a normal regulator of growth and that the microenvironment of the cell may regulate both production of MGSA and response to MGSA.

Key words: MGSA, growth factor, melanoma, autocrine

Human malignant melanoma cells have been reported to produce a variety of growth factors, including transforming growth factor-alpha (TGF_α) [1], transforming

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growth factor-beta (TGF_β), platelet-derived growth factor (PDGF) [3], fibroblast growth factor (FGF) [4], and melanoma growth stimulatory activity (MGSA) [5–7]. TGF_α, TGF_β, PDGF, FGF, and MGSA have all been suggested as possible autocrine regulators of melanoma growth. These proposed autocrine mechanisms include both positive and negative signals for growth regulation. For example, TGF_β is secreted by melanoma cells and in some instances inhibits the ability of melanoma cells to form colonies in soft agar, while other melanoma cultures have apparently lost their inhibitory response to TGF_β [9]. TGF_α has been proposed to stimulate melanoma colony formation in soft agar, but EGF/TGF_α receptor availability is quite limited in most melanoma cultures [10]. It has been postulated that EGF receptors are unavailable for binding in radioreceptor assays because the receptor is already occupied by TGF_α. Apparently, PDGF-A chain may be produced more frequently than PDGF-B chain in human melanoma, but investigators have been unable to demonstrate PDGF binding to these melanoma cells [3].

Recently, Halaban et al. have shown that antibodies to basic but not acidic FGF will inhibit the growth response of normal melanocytes to melanoma extracts [4]. We have previously demonstrated that pituitary-derived FGF produces a minimal, but significant, stimulation in melanoma cell growth [11]. However, secretion of basic FGF has not been demonstrated in melanoma cells.

An autocrine mechanism of growth stimulation has been previously demonstrated for MGSA in that: 1) MGSA released into the serum-free culture medium of the Hs294T melanoma cell line has been purified and demonstrated to stimulate the growth of Hs294T cells and exhibit specific, temperature-dependent, saturable binding to cultured Hs294T melanoma cells [8]; 2) monoclonal antibodies to MGSA inhibit the serum free growth of Hs294T melanoma cells [12].

The MGSA has been purified by gel exclusion chromatography, reverse phase high performance liquid chromatography (RP-HPLC), and preparative electrophoresis from melanoma serum-free conditioned medium. The activity resides in a family of proteins $<$ 14 to 28 Kd. The most reproducibly active form of MGSA appears to be the 16-Kd polypeptide. Cultured melanoma cells respond to 0.3–30 pM concentrations of MGSA with increased growth. Amino acid analysis of MGSA preparations suggests that MGSA is different from other previously characterized growth factors [8]. Hs294T cells do not exhibit a growth response to EGF, insulin, transferrin, progesterone, or nerve growth factor as single agents or in combination with one another [11]. Monoclonal antibody FB2AH7 to MGSA will bind to the 16-Kd form of MGSA from tumor extracts after Western blot [12], but this antibody will not bind to other growth factors. We now report the purification of MGSA from Hs294T melanoma tumors with the use of a series of gel filtration and reverse phase high performance liquid chromatography steps. These experiments demonstrate that MGSA is present in melanoma tumor tissue in sufficient quantity to recover appreciable amounts of the growth factor and that the preparative electrophoresis step previously described for purification of MGSA from conditioned medium [8] can be replaced with chromatography on a Vydak Hi-Pore C_{18} column and heparin-sepharose, with the end result of purification providing an approximately 10-fold increase in recovery of purified MGSA. We also report that though MGSA has been isolated from melanoma-conditioned medium and melanoma tumor extracts, immunoreactive MGSA is not restricted to melanoma tissue but is found in a number of proliferating tissues. Finally, in no instance did TGF_β, EGF in combination with TGF_β, PDGF, or IGF-I

mimic the growth-stimulatory activity of MGSA on Hs294T cells under the conditions of MGSA bioassay. Altogether, these data suggest that MGSA is a novel growth factor which not only has the capacity to autostimulate the growth of human melanoma cells, but may also affect the growth of other types of cells.

MATERIALS AND METHODS

Tissue culture materials and media were supplied as described previously [2]. The Hs294T cells were obtained from the American Type Tissue Culture, and a population of cells was selected which would grow on serum-free culture medium without supplementation with hormones or growth factors. Athymic nude nu/nu mice (4–5-week-old females) were obtained from the NCI Frederick Cancer Research Facility, Frederick, MD.

Tumor Formation and Extraction

Tumor formation in athymic nude mice was induced by intra-scapular injection of 1×10^6 Hs294T human melanoma cells per mouse. Palpable tumor formation was observed approximately 7 days after injection. As the tumors reached maximal size (at 24–30 days, prior to the mice becoming moribund), they were removed, minced, homogenized, and extracted with ethanolic hydrochloric acid as previously described [5].

Purification of MGSA

Bio-Gel P-30 chromatography was performed on acid ethanol extracts of tumors as described previously [7]. Fractions eluting between the ribonuclease A and the insulin molecular weight markers were pooled, lyophilized, and applied to a Waters u-Bondapak C_{18} column, eluting with a 60-min linear gradient of acetonitrile:water:TFA (6:94:0.05 to 60:40:0.05) as previously described [8]. Absorbance was monitored at 206 nm. The sensitivity range was 2.0 AUFS. The flow rate was 1.0 ml per min, and 1.0-ml fractions were collected. Fractions were lyophilized and redissolved in 100-μl of 4 mM HCl and 1/10 aliquots were assayed for recognition by the FB2AH7 antibody for MGSA in the modified dot blot enzyme-linked immunoadsorbent assay (ELISA) [12]. An ELISA-positive peak was identified that eluted from the u-Bondapak column at ~39% acetonitrile. Fractions from this peak were then re-fractionated on the Vydak Hi-Pore C_{18} column by using a 60 min linear gradient of acetonitrile:water:heptafluorobutyric acid (25:75:0.05 to 45:55:0.05). Absorbance was monitored at 214 nm and a sensitivity of 1.0 AUFS. The flow rate was 1.0 ml per min, and fractions were manually collected as peaks appeared in the chromatogram. An aliquot of each fraction was examined by SDS-polyacrylamide gel electrophoresis. The fractions eluting at 37% and 38% acetonitrile contained the 16- and 13- to 14-Kd moieties previously shown to contain MGSA bioactivity in similar preparations from Hs294T conditioned medium. Fractions corresponding to major peaks were lyophilized and re-run separately under the same conditions as above.

Heparin-Sepharose Chromatography

The RP-HPLC-purified MGSA was lyophilized, redissolved in 10 mM Tris, pH 7.5, and subjected to heparin-sepharose chromatography. A 10 × 50 mm column of heparin-sepharose CL-6B was equilibrated in 10 mM tris hydroxymethylamino-

methane (Tris), pH 7.5. After loading the partially purified MGSA onto the column, the material which did not bind to heparin-sepharose was removed by continued flow of 10 mM Tris, pH 7.5. A 200-ml linear gradient of 10 mM Tris, pH 7.5, to 10 mM Tris, 1M sodium chloride, pH 7.5, was followed by elution with 100 ml 10 mM Tris, 2M sodium chloride, pH 7.5, and finally eluted with 100 ml 6M guanidine hydrochloride. Absorbance was monitored at 280 nm, at a sensitivity of 0.1 AUFS. The flow rate was 0.5 ml/min, and 8-ml fractions were collected. Fractions were pooled based on the absorbance at 280 nm of material eluting from the heparin-sepharose, then pooled fractions were subjected to bioassay.

MGSA Bioassay and Immunoassay

The MGSA bioactivity was affirmed by using the ^{3}H-thymidine incorporation assay, with the Hs294T melanoma cells serving as the responding cell as described previously [8]. Briefly, 8,000 cells were plated in 28 × 61-mm glass scintillation vials (Wheaton #225288) in 2 ml of Ham's F-10 medium supplemented with 10% fetal bovine serum (FBS). Twenty-four hours later, cells were washed with 2 ml of phosphate-buffered saline (PBS: 8 g NaCl, 1.15 g Na_2HPO_4, 0.2 g KCl, 0.2 g KH_2PO_4, 0.1 g $MgCl_2$:$6H_2O$, 0.1 g $CaCl_2$ per liter of water, pH 7.4) and placed on serum-free F-10 medium containing HEPES (30 mM) and ovalbumin (10 μg/ml). Eight hours later ^{3}H-thymidine (5 μCi) was added to each vial and the incubation was continued for another 16 hours. The reaction was stopped by addition of methanol:ethanol (3:1). Unincorporated ^{3}H-thymidine was removed by a repeated wash with methanol, then 10 ml of counting fluid (Scintiverse II) was added to each vial, and radioactivity incorporated into DNA was counted in a Beckman liquid scintillation counter (LS-1800).

For cell number experiments, 72 hours after seeding 8 × 10^3 Hs294T cells into Wheaton scintillation vials in F-10 culture medium containing 10% fetal bovine serum, the medium was aspirated and the cells were placed on serum-free F-10 medium. Twenty-four hours later, the medium was aspirated, and dilutions of MGSA or fetal bovine serum in serum-free binding buffer (F-10 culture medium containing 30 mM HEPES and 75 μg/ml ovalbumin) were added. On the third day after growth factor additions, cells were released with trypsin, and cell number was determined from aliquots of suspended cells counted in a hemocytometer (n=4 for each point). For MGSA immunoassay, the FB2AH7 antibody was used in the modified dot blot ELISA described previously [12].

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Purified preparations of MGSA were analyzed by SDS-PAGE according to a modification of the methods of Laemmli [13]. Double strength sample buffer containing the growth factor preparation or molecular weight standards (14.4–94 Kd) was not boiled and did not contain reducing agent. The 12% polyacrylamide gels were pre-run for 1 hour prior to the addition of samples and standards. After fixing in methanol:acetic acid:H_2O (40:10:50), the gels were stained with Coomassie Blue R-250 (0.01%) and/or silver.

Newly Established Melanoma and Nevus Cultures

This study was approved by the Human Investigations Committee at Emory University. Primary cultures were established as previously described [13] from tissue

samples obtained after informed consent from melanoma patients undergoing surgical resection (without prior antitumor therapy), or from patients having nevi biopsied for histologic examination.

Monoclonal Antibodies

Monoclonal antibody FB2AH7 to MGSA has been previously characterized [12]. This antibody inhibits the in vitro growth of Hs294T cells and recognizes the 16 Kd form of MGSA in Western blot analysis.

Immunocytochemistry

At the time of subculture of melanoma and nevus cultures, cells were split such that slide chambers could be made for immunocytochemical assay and the L-dopa tyrosinase assay [15]. Nevus or melanoma cells were cultured at a density of 2.5×10^4 cells per well in two chamber/slides (Lab-tek) with 2 ml of F-10 culture medium containing 10% fetal bovine serum. After a 24-hour incubation, the cells were washed twice with PBS and then incubated with serum free F-10. After another 24 hours, slide cultures were fixed with 10.7% formalin/0.2% gluteraldehyde/0.6% Tris-buffered saline (TBS) (pH 7.5). Endogenous peroxidase was quenched by addition of 0.3% H_2O_2 in methanol for 30 min. The slides were washed in TBS for 10 min, nonspecific binding was blocked with a 1:100 dilution of normal serum (Vector Laboratories), then the slides were incubated for 30 min at 37°C with a 1:10 dilution of FB2AH7 monoclonal antibody to MGSA. As a control, a nonspecific IgM_k monoclonal antibody to alpha-lipoteichoic acid was used in place of FB2AH7. Slide chambers were then processed by using the Vectastain ABC kit according to procedures described by Vector Laboratories. The color was developed by using the peroxidase substrate, amino-ethyl-carbazole. Slides were counterstained with Mayer's hemotoxylin, washed 5 min with deionized H_2O, treated for 1 min with ammonium hydroxide, washed 5 min with running deionized H_2O, and mounted in glycerol:PBS (1:1).

Immunohistochemistry

Fixed and sectioned tissues were deparaffinized by 5-min changes of xylene; tissues were hydrated through a graded alcohol series (3 min/dilution), rinsed for 5 min in distilled water, fixed, and processed as described for immunocytochemistry.

RESULTS

RP-HPLC Purification of MGSA

Acid-ethanol extracts of melanoma tumors have been previously demonstrated to contain MGSA bioactivity [5]. In this study, acid-ethanol extracts of Hs294T tumors developed in nude mice were subjected to gel exclusion chromatography in a Bio-Gel P-30 column as previously described [8]. The Bio-Gel P-30 fractions from Pool B (lower Mr) containing MGSA bioactivity were subsequently subjected to RP-HPLC with modification of the procedure described previously for isolation of MGSA from conditioned medium, only a combination of uBondapak C_{18} and Vydak Hi-Pore columns were used. The MGSA ELISA-positive fractions eluted at ~39% acetonitrile from RP-HPLC. These fractions were pooled, lyophilized, and subjected to a second RP-HPLC step on the Vydak column eluting with a 60 min linear gradient of

acetonitrile:water:HFBA (25:75:0.05 to 45:55:0.05). Fractions eluting at 37%–38% acetonitrile (Fig. 1) contained the 16- and 13-Kd bands, respectively, previously identified as MGSA positive [9] (Fig. 2). Bioassay and ELISA assays for MGSA reconfirmed that the 16-Kd and 13- to 14-Kd containing fractions did contain the majority of MGSA. The 16 Kd moiety was found to be present primarily in those fractions eluting at 37% acetonitrile, while a 13- to 14-Kd moiety predominated in the fraction eluting at 38% acetonitrile. The 16-Kd material eluting at 37% acetonitrile and the 13- to 14-Kd material eluting at 38% acetonitrile were then separately reinjected on to the Vydak column and eluted as described earlier with the 25%–45% acetonitrile gradient and HFBA in the mobile phase. The optical density profile at 214 nm revealed a sharp peak at 37% acetonitrile (Fig. 3), and analysis of the peak by SDS-polyacrylamide gel electrophoresis revealed a single 16-Kd band as was shown in Fig. 2, lane 4. Similar results were found with the 13–14 Kd material eluting at 38% acetonitrile. The fractions eluting at both 37% (16 Kd) and 38% (13–14 Kd) acetonitrile were active in the MGSA bioassay, producing a stimulation of ^{3}H-thymidine incorporation 166% to 200% of control at concentrations in the nanogram per milliliter range.

Amino acid sequence analysis revealed that there was a unique peptide in the 16-Kd preparation. However, there was also a contaminating protein with a high percentage homology with the protein histone 2A. Contaminating histone 2A has subsequently been removed by heparin-sepharose chromatography. The more basic H2A binds more strongly to heparin-sepharose than MGSA. The MGSA bioactivity elutes from tumor extract MGSA preparations and from similar MGSA preparations derived from Hs294T conditioned medium between 0.1 and 0.3M NaCl, while histone 2A (H2A) cannot be eluted with 2M NaCl but requires 6M guanidine-HCl for elution. There was no MGSA bioactivity associated with H2A. This combined

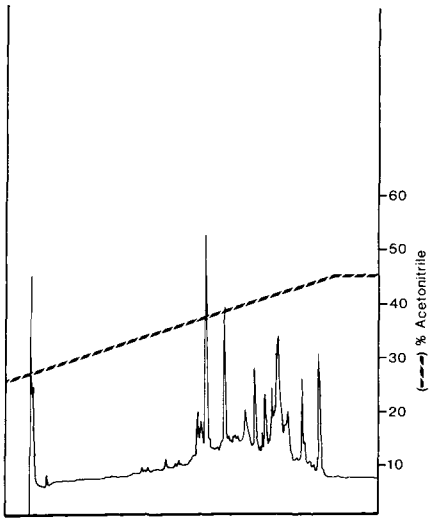

Fig. 1. RP-HPLC profile of MGSA on Vydak column. MGSA ELISA-positive fractions which eluted at ~39% acetonitrile from the u-Bondapak C_{18} column were rechromatographed on the Vydak Hi-Pore C_{18} column. A 60-min linear gradient of acetonitrile:water:HFBA (25:75:0.05 to 45:55:0.05) was employed as the mobile phase at a flow rate of 1.0 ml/min. The absorbance was monitored at 214 nm and a sensitivity of 1.0 AUFS.

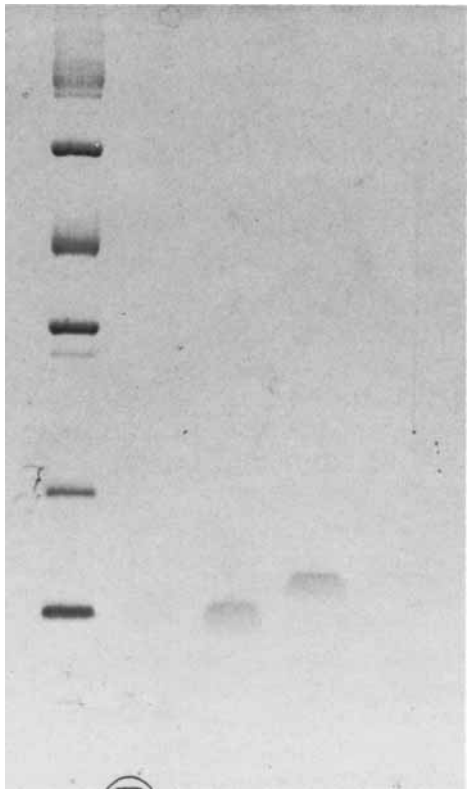

Fig. 2. SDS-PAGE of the material eluting at 37% and 38% acetonitrile from the Vydak column. A modification of the Laemmli method was employed by utilizing double strength sample buffer and omitting addition of the reducing agent to the samples or molecular weight standards. The 12% polyacrylamide gel was pre-run for 1 hour prior to the addition of samples and molecular weight standards. Lanes 1-4 consist of the following: **Lane 1**, Mr standards (phosphorylase b 94Kd, bovine serum albumin 67Kd, ovalbumin 43Kd, carbonic anhydrase 30Kd, soybean trypsin inhibitor 20.1Kd, and alpha lactalbumin 14.4Kd); **Lane 2**, blank; **Lane 3**, material eluting at 38% acetonitrile from the Vydak column; **Lane 4**, material eluting at 37% acetonitrile from the Vydak column.

RP-HPLC and heparin-sepharose chromatography procedure thus allows final purification of MGSA (Table I and Figure 4). The purified MGSA was active at concentrations of 6 to 0.06 ng/ml, producing a maximal stimulation in ^{3}H-thymidine incorporation of 171% of control and an approximately twofold increase in cell number (Fig. 5). The 5 μg of MGSA purified from 53 g of Hs294T tumor tissue contained 260,000 units, representing a recovery of 8% of the activity present in the Hs294T tumor extract. (One MGSA unit produces a stimulation in ^{3}H-thymidine incorporation into DNA half-maximal to that produced by unfractionated Hs294T-conditioned medium.)

Effects of Other Growth Factors on Hs294T Melanoma Cells

In order to determine whether other endogenous growth factors stimulate the growth of the Hs294T melanoma cells in serum-free culture medium, HPLC-purified PDGF (Collaborative Research), recombinant IGF-I (Am-Gen), and TGF$_{\beta}$ (the kind gift of Dr. Harold Moses) as a single agent and in combination with EGF (Collabo-

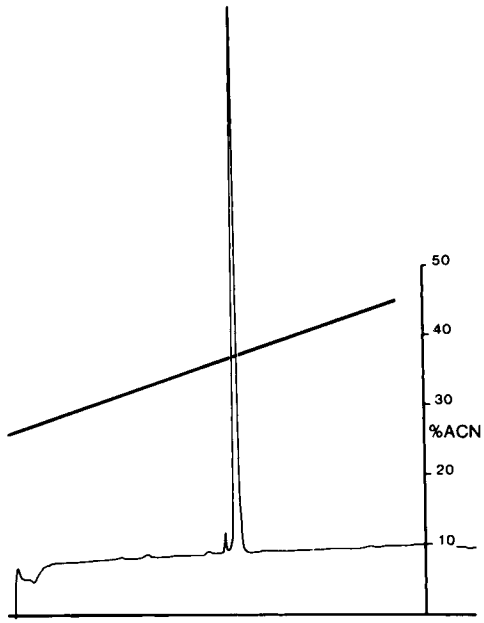

Fig. 3. RP-HPLC of 16Kd moiety. The 16 Kd material which eluted at 37% acetonitrile from the Vydak column was rechromatographed by using the 25:75:0.05 to 45:55:0.05% acetonitrile:water: HFBA 60-min linear gradient at a flow rate of 1.0 ml/min. The absorbance was monitored at 214 nm and a sensitivity of 0.5 AUFS.

TABLE I. Purification of MGSA From Hs294T Tumor Extract

Source of MGSA (Stage of Purity)	Protein content	Purification (n-fold)
Tumor tissue	53.0 g	
EtOH-HCL extraction of Hs294T tumors	289.0 mg	184
Bio-Gel P-30 Chrom. Pool B fr. 51-73	33.0 mg	1,600
RP-HPLC 6–60% ACN, 0.05% HFBA	560.0 μg	94,000
RP-HPLC Vydak C_{18} 25–45% ACN, 0.05% HFBA	53.0 μg	1,000,000
Heparin-sepharose chromatography	5.0 μg	10,000,000

rative Research, receptor grade) were assayed for MGSA-like activity. These agents were tested over a concentration range similar to that required for MGSA bioactivity in low-density Hs294T cultures. Neither PDGF or IGF-I had growth stimulatory activity for Hs294T cells under the conditions of the bioassay developed for MGSA (Fig. 4). However, less pure preparations of both of these factors from Collaborative Research have exhibited ability to occasionally, but not reproducibly, stimulate Hs294T melanoma growth at high concentrations. Since the activity was not present in more purified preparations of these growth factors, it is possible that a contaminant in these impure growth factor preparations was responsible for the stimulation of growth of the melanoma culture. The EGF and TGF_{β} had no significant effect on Hs294T cell growth, and combinations of these growth factors did not stimulate Hs294T cell growth (Fig. 4).

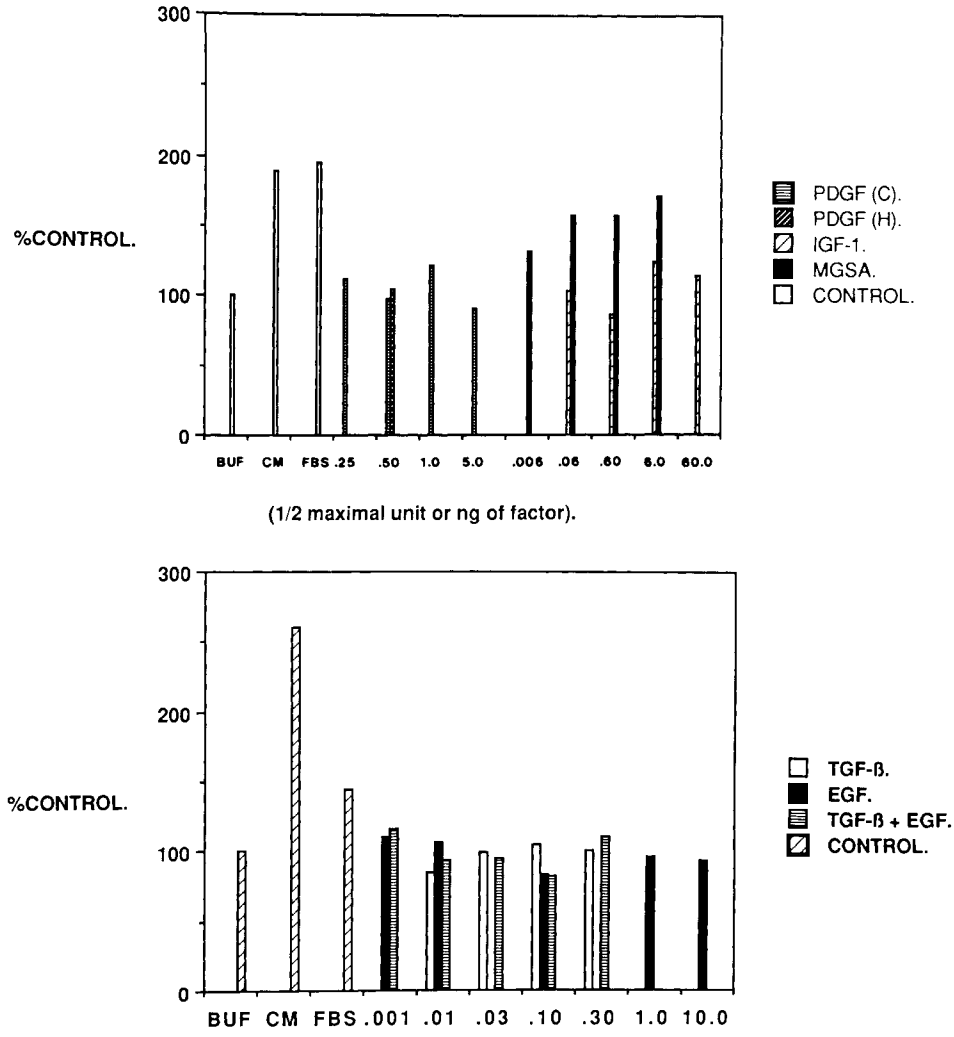

Fig. 4. Effects of other growth factors on ^{3}H-thymidine incorporation in Hs294T melanoma cells. Hs294T cells were plated into sterilized Wheaton scintillation vials in 2 ml of Ham's F-10 medium plus 10% fetal bovine serum at a density of 1×10^4 cells per vial. Cells were incubated overnight (16 hours) at 37°C, 95% air/5% CO_2, and the next day the media was aspirated, the cell layer was washed twice with serum free Ham's F-10 medium (SF-F10), and the cells were fed with 2 ml SF-F10 prior to incubation overnight. On day 3, the medium was aspirated and replaced with SF-F10 containing 20 μg/ml ovalbumin and EGF, TGF_β, PDGF, IGF-1, or purified MGSA at the concentrations indicated. Six hours later 5 μCi of ^{3}H-thymidine was added to each vial, and the cultures were incubated for an additional 18 hours. The cells were then fixed and processed for determination of radioactivity incorporated into DNA as described previously (8). Each bar represents the mean of three individual determinations except for the crude PDGF data, where each bar represents the mean of 5 separate experiments with a total of 15 individual determinations per bar. In the assays which included both TGF_β and EGF, 1 ng of EGF was included per milliliter of binding buffer. Abbreviations: **BUF**, binding buffer-control, F-10 media plus 20 μg/ml ovalbumin and 30 mM HEPES; **CM**, conditioned medium from a confluent Hs294T culture processed by low spin centrifugation; **FBS**, Ham's F-10 medium containing 1% fetal bovine serum; **PDGF (C)**, units of culture grade PDGF from Collaborative Research; **PDGF (H)**, units of HPLC grade PDGF from Collaborative Research; **IGF**, 1 ng of purified recombinant IGF-1 from AmGen; **MGSA**, 1 ng of MGSA purified by RP-HPLC and heparin-sepharose. Bars in the upper figure from left to right illustrate effects of binding buffer; conditioned medium; **FBS**; **PDGF (C)** 0.25 max units; **PDGF (C)**, **PDGF (H)** 0.5 max units; **PDGF (C)** 1.0 max units; **PDGF (C)** 5.0 max units; **MGSA** 0.006 ng; **IGF-1**, **MGSA** 0.06 ng; **IGF-1**, **MGSA** 0.06 ng; **IGF-1**, **MGSA** 6.0 ng; **IGF-1** 60 ng.

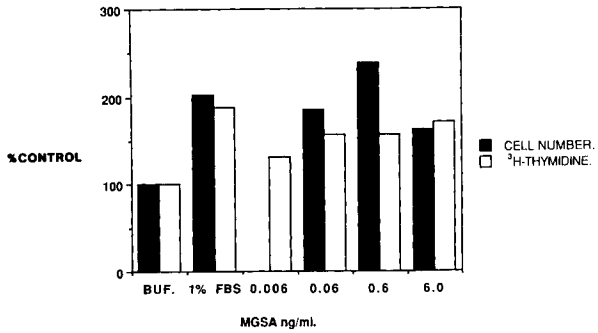

Fig. 5. Comparison of MGSA Effects on ^{3}H-Thymidine Incorporation and Cell Number. The ^{3}H-thymidine bioassay was performed on Hs294T cells as described in Figure 4. MGSA which had been purified by RP-HPLC and heparin-sepharose chromatography was assayed for growth-promoting activity on low-density Hs294T cultures at concentrations ranging from .006 ng/ml to 6 ng/ml. An *n* of four was used for each concentration and data are recorded as percent of the binding buffer control (BUF). The positive control was 1% fetal bovine serum (FBS). Cell number experiments were also performed on MGSA preparations. Briefly, 8×10^3 Hs294T cells were plated into Wheaton scintillation vials on Ham's F-10 medium containing 10% FBS. Three days later, the medium was aspirated and cells were placed on serum free F-10 medium. At 24 hours later, the medium was aspirated, and dilutions of MGSA or FBS in serum-free binding buffer (F-10 culture medium containing 30 mM HEPES and 75 μg/ml ovalbumin) were added. Controls received binding buffer alone. On the third day after growth factor additions, cells were released with trypsin, and cell number was determined from aliquots of suspended cells counted on a hemocytometer (n = 4 for each point).

Comparison of MGSA Effects on ^{3}H-Thymidine Incorporation and Cell Number

Purified MGSA is able to stimulate an increase in both ^{3}H-thymidine incorporation and cell number in low-density cultures of human Hs294T melanoma cells. Though there is some difference in the optimal concentration for maximal response between the two bioassays (optimal response with 6 ng/ml in H-thymidine bioassay versus optimal response with 0.6 ng/ml in the cell number bioassay), this difference can probably be accounted for by the differences in duration of the assays (Fig. 5).

Utilization of Monoclonal Antibody to MGSA to Evaluate the Prevalence of MGSA in Cultured Melanoma and Nevus Cells and in Fixed Tissue

Newly established cultures from human melanoma tumors and benign nevi have been examined by immunocytochemical assay for the presence of MGSA by using a monoclonal antibody to MGSA. Twenty five of 32 nevus cultures studied have been MGSA negative. In contrast, 19 of 27 melanoma cultures were positive for MGSA by immunocytochemistry (Table II, and Fig. 6). The MGSA-positive cultured nevus cells tended to be chromosomally abnormal [14]. Greater than 50% of the nevus cultures were melanin-positive and representative samples of melanin-negative nevus cultures were demonstrated to contain premelanosomes by electron microscopy.

In contrast, 10 of 15 fixed sectioned nevus tissues were MGSA positive by immunohistochemistry (Table II, and Fig. 6). These data suggest that MGSA may be involved in proliferation of nevocytes in vivo, but in vitro culture conditions are not compatible with expression of MGSA. In fixed sectioned tissue, keratinocytes and cells derived from the basal epithelium from normal skin were also MGSA positive by IHC (Table II, Fig. 6) as were three other tissues exhibiting proliferative disorders:

TABLE II. MGSA Reactivity

MGSA-negative Tissues	MGSA-positive Tissues
Metastatic adenocarcinoma (lung)	Sarcoidosis
Lymphoma	Mesangial proliferative glomerulonephritis/tubulo interstitial nephritis
Testis (normal)	
Vascular smooth muscle	Metastatic small cell anaplastic carcinoma
Fibroblasts	Basal cell carcinoma
Vascular endothelium	Cirrhotic hepatic tissue with bile ductule proliferation
Alveolar macrophages	
Lymphocytes	Melanoma (4/4)
Intestinal epithelium	Benign nevi (10)
Benign nevi (7)	
Cultures	**Cultures**
Melanoma cultures (8)	Melanoma cultures (19)
Benign nevi (25)	Benign nevi (7)[a]

[a]MGSA positive benign nevi exhibited a high incidence of chromosomal abnormality.

renal tissue exhibiting mesangial proliferative glomerulonephritis, small cell carcinoma metastatic to the liver, and sarcoidosis tissue (Table II). Associated connective tissues were MGSA negative including both cells (lymphocytes, fibroblasts, and macrophages) and fibers (collagen and elastin). In addition, alveolar macrophages, vascular endothelial cells, lung epithelium, intestinal epithelium, and testicular tissue were negative (Table II).

DISCUSSION

The substance MGSA was first described as an endogenous growth factor released into the culture medium of human malignant melanoma cells. In this study, we have determined that MGSA is not only released by cultured human melanoma tumors, but sufficient quantities of growth factor are stored in the tumor cells to enable isolation from acid-ethanol extracts of melanoma tumors. A combination of RP-HPLC and heparin-sepharose chromatography were utilized, bypassing the preparative electrophoresis step reported earlier for purification of MGSA from conditioned medium and providing a 10-fold increased yield for purified MGSA. The heparin-sepharose chromatography step enabled removal of very basic contaminants and demonstrated that MGSA differs from acidic and basic FGF as well as the endothelial growth factors which exhibit a high affinity for heparin-sepharose [16].

Studies with MGSA monoclonal antibody FB2AH7 demonstrate that approximately 70% of cultures established from human melanoma tumors are MGSA positive by immunocytochemistry while most cultures established from benign nevi were MGSA negative. Since 10 of 15 nevi were MGSA positive by immunohistochemistry when fixed sectioned tissue was studied, one might suspect that the nevocyte cultures were overgrown by fibroblasts. Several points argue against this possibility: 1) a selective low Ca^{2+} culture medium was used for the nevocyte cultures (MCDB-151), which enhances proliferation of epithelial cells and inhibits fibroblast proliferation;

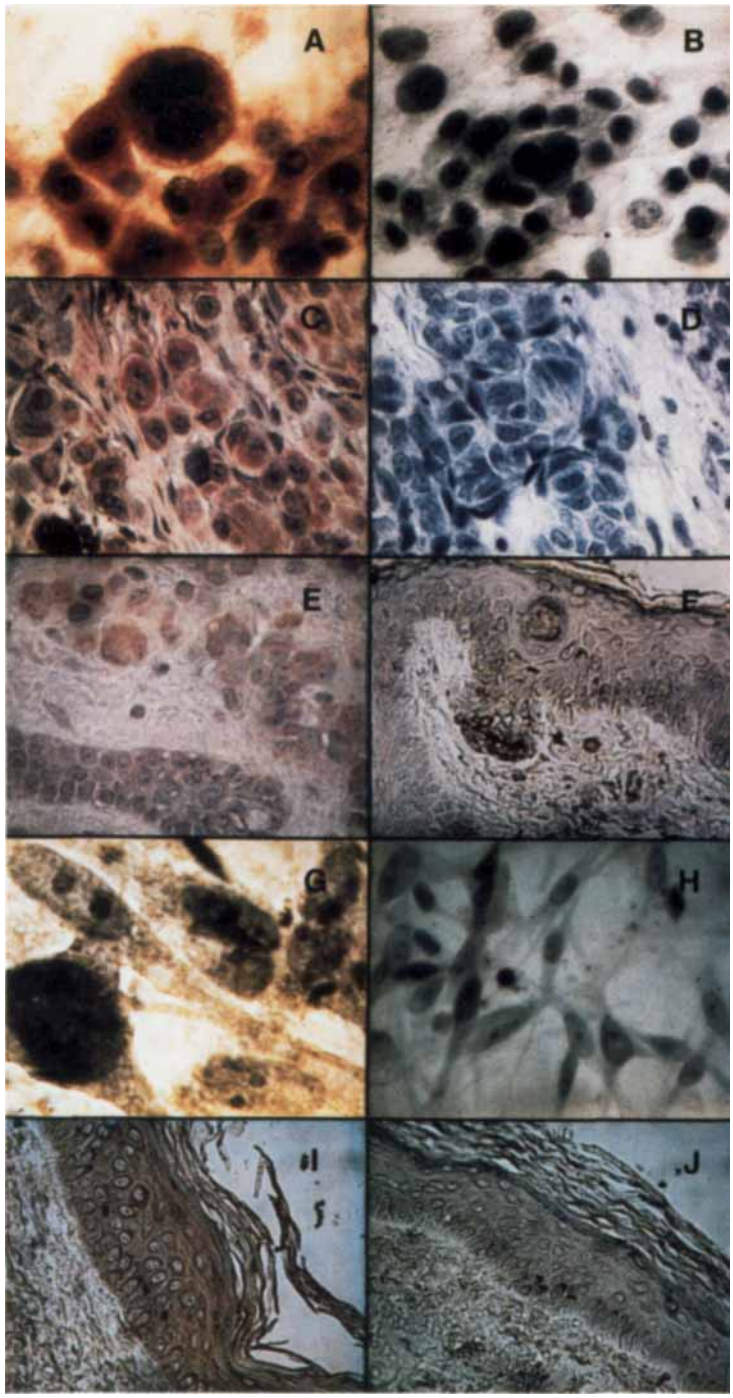

Fig. 6. Cellular localization of MGSA in cultured cells by immunocytochemistry and in fixed and sectioned tissue by immunohistochemistry. **A.** A melanoma cell culture treated with monoclonal antibody to MGSA, FB2AH7. 40× objective. **B.** A melanoma cell culture treated with non-specific monoclonal antibody. Note absence of red staining in cytoplasm. 40×. **C.** Section of a metastatic melanoma lesion treated with MGSA monoclonal antibody. 20×. **D.** Section of a metastatic melanoma lesion treated with control buffer. Note absence of red staining in cytoplasm of tumor cells. 20×. **E.** Section of a nevus lesion treated with MGSA monoclonal antibody. 20×. **F.** Section of a nevus lesion treated with control monoclonal antibody. Note absence of red staining in cytoplasm. 100×. **G.** Nevus cell culture N-48 treated with MGSA antibody. Note absence of red staining in cytoplasm. 100×. **H.** Nevus cell culture N-48 treated with control monoclonal antibody. 40×. **I.** Section of normal skin treated with MGSA antibody. 20×. **J.** Section of normal skin treated with control monoclonal antibody. Note absence of pink staining associated with epidermis. 20×.

2) electron microscopy of representative cultures of nevocytes revealed the presence of premelanosomes and/or melanosomes. Therefore, it is unlikely that the MGSA negativity in these cultures is due to failure to grow out the correct cells from the nevus tissue. Alternatively, the appropriate culture conditions (matrix molecules, growth factors, and/or nutrients) may not be present in the in vitro environment for optimal differentiation and growth. Since melanoma cells are known to produce fibronectin and specific proteoglycans in vitro which normal melanocytes do not produce in culture [17], it is possible that if the nevocytes were cultured on an appropriate substrate, then the nevocytes would be able to produce MGSA in vitro as the melanoma cultures do.

Localization of immunoreactive MGSA in the stratum spinosum of the epithelium of the skin was an unexpected finding. These data suggest that MGSA may affect the growth of a number of other cell types in addition to melanocytes. Several other tissues exhibiting proliferative disorders (small cell carcinoma of the lung metastatic to the liver, mesangial proliferative glomerulonephritis, and sarcoid tissue) also suggest that MGSA may play a role in regulation of growth of a number of tissue types, though it does appear to be most concentrated in the epithelium of the skin.

We have previously demonstrated that pituitary-derived FGF and FSH produce a minimal but significant stimulation in Hs294T cell growth. However, insulin, transferrin, progesterone, LHRH, NGF, and EGF had no effect on Hs294T cell growth as single agents or in combination [11]. We now can add IGF-1, TGF_{β}, EGF in combination with TGF_{β}, and PDGF to this list of growth factors which are not active in the MGSA ^{3}H-thymidine bioassay.

Failure of other growth factors such as PDGF, IGF-1, EGF, and TGF_{β} to mimic MGSA, along with amino acid analysis data suggesting that MGSA differs from these growth factors [8] lead us to conclude that MGSA is unlike other growth factors previously described. In addition the data shown here demonstrate that the heparin-sepharose binding capacity of MGSA differs from that of acidic or basic FGF. The MGSA appears to contribute to the relatively autonomous growth of malignant melanoma cells, and, based on localization of immunoreactive MGSA in other proliferating tissues, this growth factor may also serve as a mitogen for a number of other cell types.

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