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Characterization of the Role of Melanoma Growth Stimulatory Activity (MGSA) in the Growth of Normal Melanocytes, Nevocytes, and Malignant Melanocytes

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Melanoma growth stimulatory activity (MGSA) was originally described as an endogenous growth factor for human melanoma cells. To test the hypothesis that an MGSA autocrine loop is responsible for the partial freedom from growth control observed in nevocytes and melanoma cells, MGSA growth response and MGSA mRNA/protein levels were examined in these cells compared with normal melanocytes. As a single agent, or in combination with other factors, MGSA stimulated the growth of normal human epidermal melanocytes as well as other growth promoters for melanocytes. Nevocytes were not as responsive to exogenous MGSA as melanocytes. MGSA mRNA was minimal or not detected in cultured normal melanocytes, although the protein was present when the cells were cultured in the presence of serum/growth factors and absent when serum/growth factors were omitted. In contrast, MGSA mRNA was constitutively expressed in the absence of exogenous growth factors in cultures established from benign intradermal and dysplastic nevi and melanoma lesions in different stages of tumor progression. Nevus cultures contained immunoreactive MGSA protein in the presence of serum but were negative or only faintly positive in the absence of serum. Melanoma cell lines were positive for MGSA protein in both the presence and the absence of serum. Thus, continued expression of both MGSA mRNA and MGSA protein in the absence of exogenous hormones or serum factors may correlate with partial freedom from growth control exhibited by malignant melanocytes.

Key words: expression, deregulation, tumor progression

Abbreviations: bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CM, conditioned medium; CsCl, cesium chloride; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; FBS, fetal bovine serum; FSH, follicle-stimulating hormone; IB15, cyclophilin gene cDNA; IGF-1, insulin-like growth factor-1; MGSA, melanoma growth stimulatory activity; mRNA, messenger ribonucleic acid; PDGF, platelet-derived growth factor; rMGSA, recombinant melanoma growth stimulatory activity; RNA, ribonucleic acid; SDS, sodium dodecyl sulfate; TPA, 12-O-tetradecanoyl phorbol-13-acetate.

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It has been postulated that the release from dependence on exogenous growth factors in many transformed cells results from the endogenous production of growth factors, some of which may act as autocrine growth stimulants [1]. Metastatic melanoma cell lines often grow in serum-free culture medium without addition of exogenous growth factors [2]. These cells produce a number of endogenous growth factors, one of which is melanoma growth stimulatory activity (MGSA). MGSA was originally purified from serum-free conditioned media of the human melanoma cell line Hs294T. MGSA appears to act as an autocrine growth factor for the Hs294T melanoma cell line in that 1) MGSA is produced by Hs294T melanoma cells [3,4]; 2) MGSA stimulates ^{3}H-thymidine incorporation and increase in cell number in Hs294T melanoma cells [5,6]; and 3) antibodies to MGSA inhibit the growth of Hs294T cells based on both ^{3}H-thymidine incorporation and cell number data [7]. These experiments were performed with a clonally selected subpopulation of Hs294T cells, which grow for an extended time on serum-free culture medium. The data suggest that the MGSA generated by the Hs294T cell population is used by that same population for growth. Although one cannot rule out the possibility of heterogeneity due to clonal drift or the possibility that MGSA is produced in one stage of the cell cycle while cells in a different stage of the cycle respond to MGSA, the data support an autocrine role for MGSA.

Immunohistochemical studies using monoclonal antibodies to MGSA have shown that several tissues contain immunoreactive MGSA, including benign nevi, melanoma in situ, and metastatic melanoma lesions [3]. Immunocytochemical studies revealed that metastatic melanoma cells continue to express MGSA in vitro even in the absence of serum or other exogenous growth factors [3]. These data led to two hypotheses: 1) that MGSA may play a role in the normal growth control of the melanocyte and 2) that, during the escape from growth control during melanoma tumor progression, the MGSA gene may be deregulated, allowing for continued MGSA transcription and translation. Experiments described here were designed to examine these hypotheses by 1) measuring the growth response of normal melanocytes and benign nevocytes to MGSA in vitro and comparing the effects of MGSA on melanocyte growth to other agents previously reported to stimulate melanocyte growth and 2) comparing the level of MGSA mRNA and MGSA protein in normal melanocytes, in nevi in varying stages of tumor progression, and in melanoma cell lines cultured in the presence or absence of serum. The results of the work described here confirm that MGSA can play a role in the growth control of normal melanocytes and suggest that, whereas both nevocytes and malignant melanocytes express elevated levels of MGSA mRNA compared with normal melanocytes, only malignant melanocytes continue to express detectable levels of MGSA protein in the absence of serum or exogenous growth factors.

MATERIALS

MGSA was obtained from the conditioned medium (CM) of Hs294T cells as described previously [6], or for some experiments recombinant MGSA (rMGSA) was purified from CM from the CHO-MGSA expressing clone [8]. The phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA), forskolin, and dibutyryl cAMP were purchased from Sigma Chemical Company (St. Louis, MO). Melanocyte growth medium, bovine pituitary extract, and human epidermal melanocytes were purchased

from Clonetics (San Diego, CA). MCDB-151 culture medium was purchased from Sigma. Basic fibroblast growth factor (bFGF) was purchased from Collaborative Research Inc. (Bedford, MA) as bovine bFGF (over 95% pure). Insulin-like growth factor-1 (IGF-1) was purchased from AMGen Biologicals (Thousand Oaks, CA) as recombinant human IGF-1 (over 99% pure). The melanoma cell lines Hs294T, Sk-Mel-2, Sk-Mel-5, Sk-Mel-24, Sk-Mel-31, WM-115, and WM-266-4 were obtained from the American Type Culture Collection. Melan-a cells were a kind gift from Ian R. Hart (Imperial Cancer Research Fund, England).

METHODS

^{3}H-Thymidine Studies

Nevocytes. Nevus cultures were established from nevi according to our standard procedures [4]. The pathology reports on each of the nevi were as follows.

N-73, benign intradermal melanocytic nevus

N-108, predominantly melanocytic nevus (epidermal) with a lentiginous component

N-112, benign intradermal melanocytic nevus

N-115, benign intradermal melanocytic nevus (possibly a congenital melanocytic nevus)

N-116, atypical compound melanocytic nevus with marked dysplasia

Cells were maintained in culture on MCDB-151 medium plus 20% fetal bovine serum for 4–12 passages during the course of these studies. For nevocyte growth bioassay, N-108 cells were used. These cells have relatively high levels of MGSA mRNA and contain immunoreactive MGSA in the presence of serum. N-108 cells were released with trypsin/EDTA and plated into Wheaton glass scintillation vials at a density of 8,000 cells per vial in MCDB medium supplemented with epidermal growth factor (EGF) (10 ng/ml), bovine pituitary extract, insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), antibiotics, and antimycotics plus 10% fetal bovine serum (FBS). The next day the growth medium was aspirated, cells were washed once with MCDB-151 medium and placed in 2 ml serum-free MCDB medium. Twenty-four hours later, the medium was aspirated, and growth supplements were added in fresh MCDB medium. After incubation for 6 hr, 2.5 μCi ^{3}H-thymidine were added per vial and cells were incubated for 18 hr at 37°C in an atmosphere of 5% CO_2/95% air. The cells were then fixed with methanol/ethanol (3:1) and unincorporated ^{3}H-thymidine was removed prior to liquid scintillation counting as described previously [3].

Melanocytes. Human melanocytes purchased from Clonetics were maintained in melanocyte growth medium (Clonetics) containing 5 μg/ml of insulin, 0.5 μg/ml of hydrocortisone, 10 ng/ml of TPA, 1 ng/ml recombinant bFGF, bovine pituitary extract, penicillin, streptomycin, and amphotericin-B. For bioassay, cells were trypsinized and replated into 24 well plates (Corning) at a density of 2,500 cells per well. Cells were cultured for 4 days refeeding every 48 hr. On day 5, the culture medium was aspirated and replaced with MCDB-151 medium containing 1% FBS. Thirty-six hours later, the medium was aspirated and replaced with MCDB-151 medium containing 10 μg/ml

BSA with or without combinations of growth supplements (bFGF, insulin, MGSA, TPA). After incubating for 6 hr, 2.5 μCi ^{3}H-thymidine were added per well and cells were incubated for 18 hr at 37°C in an atmosphere of 5% CO_2/95% air. The medium was then aspirated, and cells were fixed with 10% ice-cold TCA, washed thrice with 5% cold TCA, and solubilized in 0.5 N NaOH (1 hr at 37°C) prior to scintillation counting.

Immunocytochemical studies. Immunocytochemical localization of MGSA was performed using the FB2AH7 monoclonal antibody to MGSA as previously described [3,7]. The control mouse IgM antibody was to α-lipotechoic acid.

RNA Studies

Cell culture. The human malignant melanoma cell lines Hs294T, Sk-Mel-2, Sk-Mel-5, Sk-Mel-24, Sk-Mel-31, WM-115, and WM-266-4 were cultured in Eagle's minimal essential medium: F-12 medium (1:1), supplemented with 0.1 mM nonessential amino acids, 2 mM L-glutamine, penicillin, streptomycin, and Fungizone. Stock cultures were maintained in this medium with the addition of 10% FBS. Experimental melanoma cultures were examined both with and without 10% FBS. Nevus and melanocyte cultures were maintained as described above.

RNA Extraction. A 4 M solution of guanidine isothiocyanate was added directly to the monolayer culture. Total RNA was isolated by centrifugation through a cushion of 5.7 M cesium chloride (CsCl). Reextraction of RNA with phenol/chloroform was then followed by ethanol precipitation [9].

Northern blot analysis. Twenty micrograms of total RNA were denatured at 65°C in the presence of formamide and formaldehyde, subjected to electrophoresis on a 1.2% agarose/formaldehyde gel, and blotted to nitrocellulose filter. Hybridizations were performed under high stringency conditions. The 0.73 kbp EcoR1 fragment of the MGSA cDNA insert isolated from a human term placenta cDNA library was used as a hybridization probe as described previously [10]. The membranes used for hybridization with the cDNA MGSA probe were subsequently washed with 0.2 × SSC/0.1% SDS at 52°C, for 1 hr with several changes of the buffer, and immediately rehybridized with the ~0.7 kbp Pst1/EcoR1 fragment of the 1B15 cDNA insert encoding cyclophilin, the binding protein for cyclosporine (the 1B15 gene is expressed at the same level throughout the cell cycle and can be used to test for equal loading of total RNA on the gel [11]. The washed, hybridized membranes were exposed to XAR-5 Kodak films for 24 hr to obtain the autoradiographic scan.

Dot blot analysis. Twenty micrograms of total RNA were denatured at 65°C in the presence of formamide and formaldehyde and then spotted on nitrocellulose filter in amounts ranging from 1.25 μg to 10 μg per sample. Hybridization was performed as described for Northern analysis using the p_1MGSA and 1B15 probes.

Scanning laser densitometry. An LKB 2002 UltroScan laser densitometer and the LKB 2190-001 GelScan Apple II computer software package were used to evaluate the autoradiographic signal on XAR-5 Kodak films of the nitrocellulose membranes hybridized with the p_1MGSA or 1B15 probes. The scan speed used was 100 mm/min, and the absorbance ranged between 2 and 4, according to the intensity of the signal. The peaks obtained were integrated in a single area, after background subtraction and baseline determination. MGSA expression was normalized by comparing lane variation in 1B15 expression to correct for variations in RNA loading. Data are displayed by histogram.

RESULTS

Effect of MGSA and Other Growth Factors on Melanocyte and Nevocyte Growth

The growth in vitro of normal human melanocytes from newborn foreskin or adult skin is stimulated by a number of growth promoters, including cholera toxin and the phorbol ester TPA [12], dibutyryl cAMP, and bFGF [13] as well as insulin, transferrin, EGF, bovine pituitary extract, and serum [14–16]. Although melanocytes appear to require TPA or bFGF and certain other additives for an expanded life span in culture and maintenance of the dendritic phenotype, nevus cells do not require TPA for growth and bFGF concentrations can be greatly reduced or omitted without a total loss of proliferative capacity [see 14, 17 for review]. Melanoma cells are relatively independent of exogenous growth factors but can respond to exogenous FGF and FSH [18]. In fact, TPA inhibits the growth of some melanoma cells [15,17]. Basic FGF can replace TPA in melanocyte cultures [13]. Therefore, in examining the mitogenic effects of MGSA on melanocytes and nevocytes, it was important to compare it to other factors that stimulate melanocyte growth and to examine factors that might work in combination with MGSA.

Comparison of the effects of MGSA and other growth stimulators on melanocytes or nevocyte growth revealed that MGSA is comparable to bFGF and insulin in its ability to stimulate the growth of melanocytes as measured by ability to stimulate the incorporation of ^{3}H-thymidine into DNA (Table IA,B). For most data points, several experiments were performed, with triplicate cultures for each experimental value. As single agents, dibutyryl cAMP, insulin, and MGSA stimulated about a twofold increase in ^{3}H-thymidine incorporation into melanocytes cultured in basal MCDB 151 medium, with the response to cAMP being greater than insulin or MGSA. As single agents, neither TPA (data not shown) nor bFGF (50 or 10 ng/ml) significantly stimulated melanocytes (Table IA). Combinations of either MGSA or bFGF with insulin and TPA had a greater stimulatory effect than any of the growth factors alone (Table IA). When normal human melanocytes were cultured with the additives insulin and TPA, they were more sensitive to MGSA such that tenfold lower concentrations

TABLE IA. Effects of MGSA and Other Growth Modulators on the Growth of Cultured Melanocytes

	rMGSA (ng/ml)				
	0	.006	.06	.6	6
Binding buffer	100 (6)[a]	113 ± 1 (4)	184 ± 2 (6)	175 ± 3 (6)	152 ± 3 (6)
Insulin (5 μg/ml)	187 ± 4 (5)	205 ± 11 (4)	210 ± 10 (4)	321 ± 17 (4)	194 ± 7 (4)
IGF (6 ng/ml)	130 ± 9 (1)	—	147 ± 5 (1)	261 ± 22 (1)	—
Dibutyryl cAMP (1 mM)	215 ± 3 (1)	—	230 ± 10 (1)	177 ± 7 (1)	162 ± 12 (1)
Forskolin (50 μm)	142 ± 7 (1)	—	—	—	—
bFGF					
50 ng/ml	131 ± 3 (5)	—	—	—	—
10 ng/ml	110 ± 6 (5)				
Insulin (5 μg/ml)/TPA (10 ng/ml)	183 ± 6 (4)	306 ± 14 (3)	316 ± 17 (4)	224 ± 8 (4)	223 ± 8 (4)
Insulin/TPA/bFGF	302 ± 15 (4)	—	—	—	—
Melanocyte growth medium	701 ± 21 (6)	—	—	—	—

[a]CPM ^{3}H-thymidine incorporated into DNA expressed as percent binding buffer control ± SEM. Number in parentheses = number of separate experiments performed, with triplicate samples in each experiment.

TABLE IB. Effects of MGSA and Other Growth Modulators on the Growth of Cultured Nevocytes

	rMGSA (ng/ml)			
	0	.06	.6	6
Binding buffer	100 (2)[a]	120 ± 8 (2)	157 ± 12 (2)	124 ± 6 (2)
IGF-I (6 ng/ml)	131 ± 2 (2)	156 ± 3 (2)	—	173 ± 7 (2)
dibutyryl cAMP (1 mM)	100 ± 1 (3)	104 ± 5 (2)	138 ± 6 (2)	183 ± 4 (2)
IBMX (0.1 mM)	104 ± 1 (3)	—	170 ± 6 (2)	—
Forskolin (50 μM)	99 ± 4 (3)	—	—	—
bFGF				
500 pg/ml	193 ± 2 (1)	—	—	—
100 pg/ml	190 ± 7 (1)			
50 pg/ml	122 ± 5 (1)			
FBS (1%)	300 ± 7 (2)	—	—	—
TPA 10 ng/ml	113 ± 4 (2)		103 ± 6 (2)	

[a]CPM ^{3}H-thymidine incorporated into DNA expressed as percent binding buffer control ± SEM. Number in parentheses = number of separate experiments performed, with triplicate samples in each experiment.

of MGSA provided maximal stimulation of ^{3}H-thymidine incorporation into DNA. The effect of insulin (5 μg/ml) and MGSA (0.6 ng/ml) was nearly additive (321% of the binding buffer control compared with the expected additive value of 362%). In some instances the dose response to MGSA was shifted up (dibutyryl cAMP) or down (TPA and insulin) in the presence of other growth factors.

Melanocyte growth medium has numerous additives that can significantly alter cell growth. For melanocyte growth medium, this includes insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), TPA (10 ng/ml), bFGF (1 ng/ml), and bovine pituitary extract. Melanocyte growth medium stimulated ^{3}H-thymidine incorporation into DNA in melanocytes sevenfold over the MCDB-151 basal medium alone. MGSA alone partially replaced this combination of additives (1.84-fold stimulation with 0.06 ng/ml MGSA) whereas MGSA, TPA, and insulin increased this to a 3.16-fold stimulation. This is still less than the complete melanocyte growth medium but is equal to threefold stimulation which occurred with the combination of bFGF, TPA, and insulin.

In general, cultured nevocytes were less responsive than melanocytes to MGSA (Table IB). In nevocytes and melanocytes, IGF-1 slightly stimulated ^{3}H-thymidine incorporation and had an additive effect on MGSA stimulation of ^{3}H-thymidine incorporation. TPA had no significant effect, and TPA in combination with MGSA (0.6 ng/ml) was not stimulatory. Forskolin, isobutylmethyl xanthine, and dibutyryl cAMP had no effect on nevocyte growth or MGSA bioactivity and did not significantly enhance the response of nevocytes to MGSA. However, under the conditions of this assay, ^{3}H-thymidine incorporation in serum depleted normal melanocytes was stimulated by dibutyryl cAMP or forskolin, and neither MGSA nor bFGF could enhance the cAMP effect.

MGSA Expression in Cultured Melanocytes

To examine the hypothesis that expression of MGSA mRNA might be progressively deregulated as melanocytes progress toward malignancy, we compared the endogenous expression of MGSA mRNA by Northern and dot blot analyses in normal melanocytes; nevocyte cultures established from benign intradermal, lentiginous, and dysplastic nevi; and malignant melanoma cell lines. We also examined intracellular

MGSA protein by immunocytochemistry using the FB2AH7 monoclonal antibody to MGSA previously described [7].

Immortalized normal human melanocyte cell lines are not available for comparison of MGSA expression to immortalized human melanoma cell lines. However, primary human melanocyte cultures are available from Clonetics, and one mouse melanocyte cell line has been developed. Therefore, MGSA mRNA expression was analyzed in two Clonetics human ectodermal melanocyte cultures grown in normal melanocyte growth medium as described in Methods and in the same basal MCDB151 medium without the addition of the phorbol ester TPA. MGSA expression was also examined in the immortalized mouse melanocyte Melan-a cell line [18]. After repeated analysis, no MGSA mRNA expression could be detected in the human melanocytes in the presence or absence of TPA (Fig. 1). Two different molecular weight transcripts of the non cell cycle-regulated 1B15 gene were observed, one of them exhibiting a mobility slightly less than for the control cell line Hs294T. The mouse melanocyte cell line Melan-a exhibited a barely detectable level of MGSA mRNA after a 6 day exposure, which further demonstrated that melanocytes do not exhibit the same level of MGSA mRNA expression seen in nevi and melanoma. However, immunocytochemical studies of normal human epidermal melanocytes did reveal that, when melanocytes are cultured in the presence of 10% FBS, immunoreactive MGSA is present in these cells. Thus we can speculate that the MGSA mRNA coding for this MGSA protein is rapidly degraded in normal human epidermal melanocytes (NHEM) cultured either in the presence or absence of the TPA, bFGF, insulin, and bovine pituitary extract included in melanocyte growth medium. In contrast, the MGSA protein is quite stable when the cells are cultured in melanocyte growth medium or Hams F-12 medium plus serum, but MGSA protein is absent when growth factors/serum are omitted from the culture medium.

MGSA Expression in Cultured Nevocytes

Prior immunohistochemical studies of human nevocytes cultured in serum-free medium revealed that little or no MGSA was present in these cultures unless there were chromosomal abnormalities [3,4]. In contrast, about 70% of the metastatic melanoma cultures were MGSA positive based on immunocytochemistry. Since the expression of the mouse homolog of the human MGSA gene KC has been shown to be induced by serum, PDGF [19,20], and several other mediators of growth and the inflammatory response [21,22], we wanted to determine whether MGSA mRNA expression might be elevated in response to serum in cultures established from nevi as compared to melanoma cell lines. MGSA mRNA expression was analyzed in five nevus cultures and in seven melanoma-derived cell lines under serum-free and serum-containing conditions (Figs. 2, 3). All the cell lines expressed the $\sim$1.1 kb mRNA for MGSA in both the presence and the absence of serum. In three of the five nevus cell lines, the MGSA mRNA expression was higher in serum-free culture medium as compared with medium containing serum (Fig. 2). The nevus culture showing the highest level of MGSA mRNA when serum was present was N-116, an atypical compound melanocytic nevus showing marked dysplasia. However, this nevus had the lowest endogenous mRNA in the absence of serum. The nevus exhibiting the lowest level of MGSA mRNA in the presence of serum and the highest endogenous level of expression in serum-free medium was N-112, a benign intradermal melanocytic nevus. Constitutive expression of MGSA in the absence of serum factors may

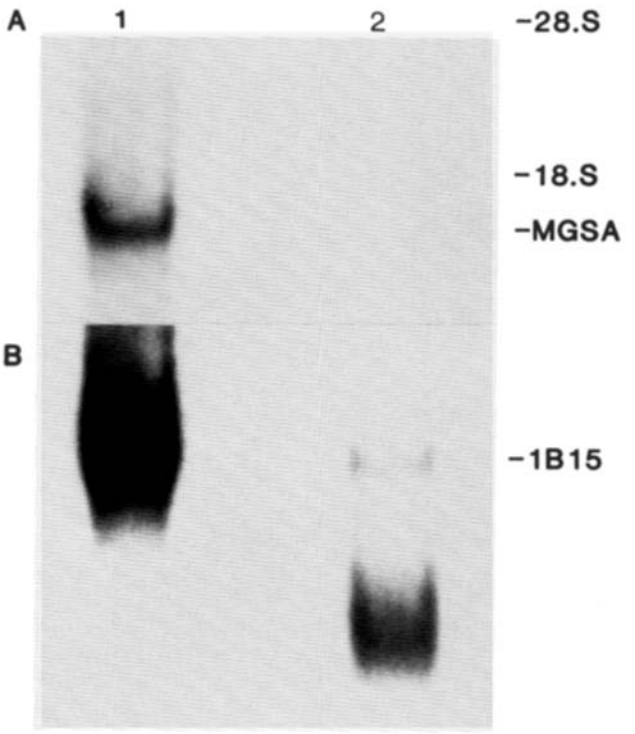

Fig. 1. Northern blot analysis of MGSA mRNA in normal human cutaneous melanocytes grown in normal melanocyte growth medium (MGM). Cellular RNA was extracted by CsCl centrifugation and ethanol precipitation; 20 μg of total RNA per sample were electrophoresed on 1.2% agarose/formaldehyde gel. Levels of MGSA mRNA were determined by Northern blot analysis. The 0.73 kbp EcoR1 fragment of the MGSA cDNA insert was used as a hybridization probe **(A).** The ~0.7 kbp Pst1/EcoR1 fragment of the 1B15 cDNA insert encoding cyclophilin, the binding protein for cyclosporine, was used as a hybridization probe, to rehybridize the membrane **(B).** Hybridization was performed under high stringency conditions as described in Methods. The washed, hybridized membrane was exposed to XAR-5 Kodak film for 24 hr to obtain the autoradiographic scan. A: Comparison of MGSA mRNA levels in the human melanoma cell line Hs294T (lane 1) grown in 10% fetal bovine serum (FBS) containing medium, and the normal human ectodermal melanocyte-derived cell line NHEM-27 grown in normal melanocyte medium (MGM) (lane 2). B: Determination of 1B15 gene mRNA levels in the same cell lines displayed in A. The position of the 28-S and 18-S bands of the ethidium bromide stain of a duplicate gel is indicated at left.

occur prior to, coincident with, or after dysplasia occurs and that even dysplastic nevi may continue to respond to serum with enhanced MGSA mRNA levels.

Due to technical problems, we were unable to keep all five of the nevi in culture for MGSA immunocytochemistry. The two cultures studied (N-73 and N-108) were MGSA positive in the presence of serum but negative to faintly positive in the absence of serum (Table II).

MGSA Expression in Cultured Melanoma Cells

When the MGSA mRNA expression in melanoma cells cultured with serum was compared with MGSA mRNA expression in the absence of serum, all of the melanoma cell lines, except for the Hs294T cell line, expressed more MGSA mRNA

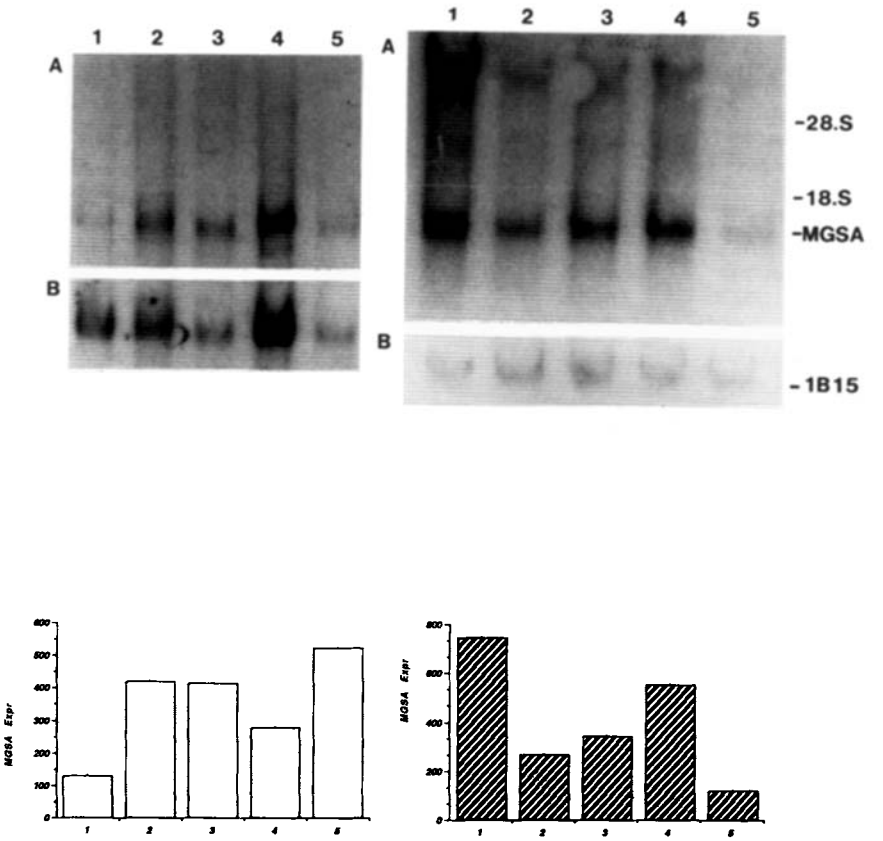

Fig. 2. Northern blot analysis of MGSA mRNA in nevus cell lines grown in serum free (SF) medium. Cellular RNA was extracted and processed as described for Figure 1 and in Methods. **A:** Comparison of MGSA mRNA levels in the nevus cell lines. **Left:** MGSA mRNA levels in nevus cultures grown in serum free medium, N-116 **(lane 1),** N-108 **(lane 2),** N-73 **(lane 3),** N-115 **(lane 4),** and N-112 **(lane 5),** grown in SF medium. **Right:** MGSA mRNA levels in the nevus cell cultures grown in serum-containing medium: N-116 **(lane 1),** N-108 **(lane 2),** N-73 **(lane 3),** N-115 **(lane 4),** and N-112 **(lane 5). B:** Determination of the 1B15 gene mRNA levels in the same nevus cell lines displayed in A, showing nevus cells cultured in serum free medium on the left (1–5) and serum containing medium on the right (1–5). The position of the 28-S and 18-S bands of the ethidium bromide stain of a duplicate gel is indicated at left. **Bottom.** Bar graphs displaying the results of normalization of MGSA signal intensity to 1B15 by scanning laser densitometry. Open bars, serum free medium; cross-hatched bars, serum containing medium.

in the absence of serum than in its presence (Fig. 3). When the seven cell lines were analyzed as a group, regardless of the culture conditions, the highest expression of MGSA mRNA was observed in the Hs294T cell line, followed by Sk-Mel-5, Sk-Mel-2, WM-266-4, WM-115, Sk-Mel-31, and Sk-Mel-24 (Fig. 3). Thus most melanoma cells constitutively produce MGSA even without addition of exogenous growth or serum factors.

Immunocytochemical studies of the melanoma cell lines revealed that immunoreactive MGSA was present in serum-free as well as serum-stimulated cultures. All six of the cell lines were strongly positive under both sets of culture conditions, suggesting

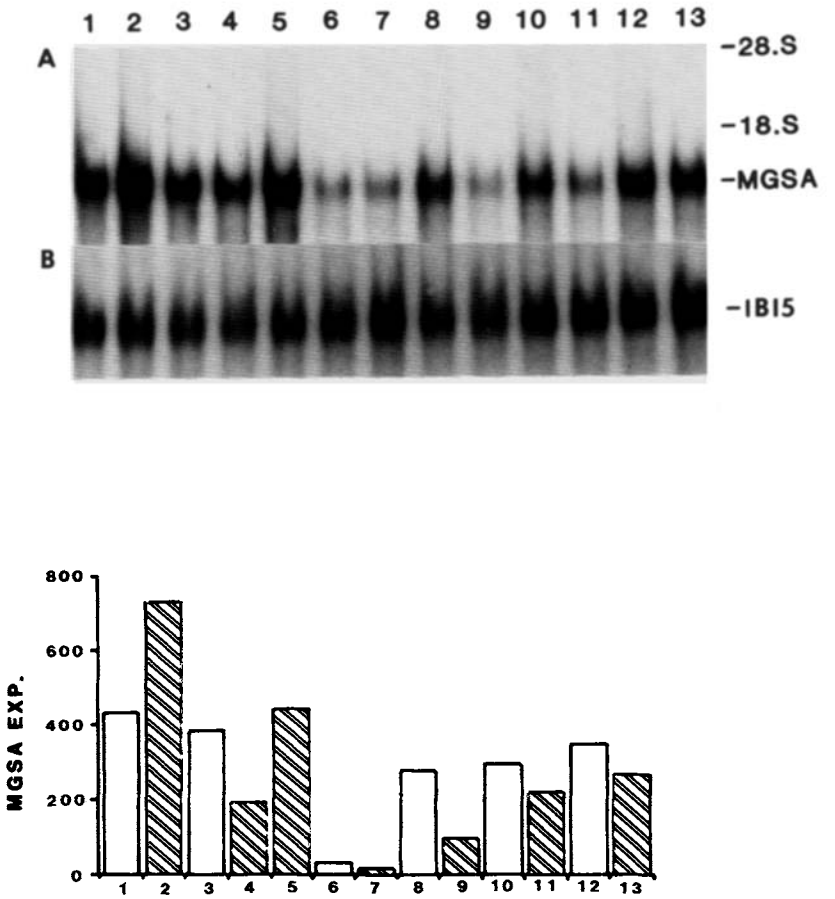

Fig. 3. Northern blot analysis of MGSA mRNA in human melanoma cell lines grown in serum-free (SF) medium and in serum-containing (S) medium. Cellular RNA was extracted and processed as described for Figure 1 and in Methods. **A:** Comparison of MGSA mRNA levels in the human melanoma cell lines: Hs294T in SF and S containing medium **(lanes 1, 2),** Sk-Mel-2 in SF and S medium **(lanes 3–4),** Sk-Mel-5 in S medium **(lane 5),** Sk-Mel-24 in SF and S medium **(lanes 6, 7),** Sk-Mel-31 in SF and S medium **(lanes 8, 9),** WM-115 in SF and S medium **(lanes 10, 11),** and WM-266-4 in SF and S medium **(lanes 12, 13). B:** Determination of 1B15 gene mRNA levels in the same human melanoma cell lines and in the same growth conditions displayed in A. The position of the 28-S and 18-S bands of the ethidium bromide stain of a duplicate gel is indicated at left. **Bottom.** Bar graph displaying the results of normalization of MGSA signal intensity to 1B15 by scanning laser densitometry showing cells cultured in serum free medium (open bars) or serum-containing medium (cross-hatched bars).

that melanoma cells continue to translate the MGSA mRNA even in the absence of serum (Table II).

Dot blot analysis of varying concentrations of nevus and melanoma RNA from cells cultured in the presence or absence of serum demonstrated the mean levels of expression of MGSA (normalized to 1B15) were comparable in the nevus cultures and in the melanoma cultures (nevus: serum free = 351, serum plus = 400; melanoma: serum free = 289, serum plus = 275).

TABLE II. Immunocytochemical Localization of MGSA Protein

Culture	MGSA immunoreactivity[a]	
	With serum/ growth factors	Without serum/ growth factors
Normal human epidermal melanocytes	++	–
N-73	++	–
N-108	++	–
Hs294T	+++	+++
Sk-Mel-2	+++	+++
Sk-Mel-5	+++	+++
Sk-Mel-24	+++	+++
Sk-Mel-31	+++	+++
Wm-115	+++	+++
Wm-266-4	+++	+++

[a]The relative MGSA immunoreactivity was ranked based on degree of AEC substrate reaction product.

DISCUSSION

Normal melanocytes are more responsive to the growth-promoting effects of exogenous growth factors and growth promoters than nevocytes or malignant melanocytes. Since the nevocyte and malignant melanoma cells produce and respond to the growth factor MGSA, the diminished response of these cells to exogenous growth factors and promoters may be associated with the prior stimulation by the endogenous MGSA. In the work described here, we have shown that, as a single agent, MGSA augments melanocyte growth, and in combination with insulin and TPA it is as potent a mitogen as bFGF, insulin, and TPA in combination. Both MGSA and TPA are potent inducers of MGSA mRNA expression in a number of cell systems [10,21,22], and we have also observed that TPA and IGF-1 induce MGSA mRNA expression in melanoma cells (unpublished work). Thus the additives that augment melanocyte growth may accomplish this by inducing expression of MGSA mRNA.

Constitutive expression of MGSA mRNA and/or protein in the absence of exogenous serum or growth factors appears to be a very early event in the progression of normal cutaneous melanocytes to malignant melanoma. MGSA mRNA is not detectable in normal human melanocytes growing in defined culture medium, and the MGSA protein is present only when these cells are cultured with exogenous growth factor or serum. The inability to detect MGSA mRNA in normal human melanocytes cultured in melanocyte growth medium was not due to a down regulation of MGSA expression because of prolonged culture with phorbol ester, since even when the NHEM-27 cells were cultured in absence of TPA, no MGSA mRNA expression was detected. In the Melan-a mouse melanocyte cell line, MGSA mRNA levels were quite low, requiring a 6 day exposure of the Northern blot to detect a faint band. The AUUUA repeats in the 3′-untranslated region of the MGSA mRNA are thought to signal for rapid degradation of the mRNA, so the low mRNA level in cycling cells is not an unexpected finding.

The unexpected finding is that MGSA mRNA is expressed at fairly high levels in both nevus and melanoma cells in both the presence and the absence of serum. The data presented here suggest that, in nevus and melanoma cells, MGSA mRNA expression may be constitutive. Thus, when growth factors are limiting, nevocytes and

some melanoma cells may continuously express MGSA mRNA. MGSA mRNA levels are reported to be elevated in a number of tumor types, and this elevation has been attributed to both increased transcription and decreased degradation of the MGSA mRNA [10,23,24]. In nevi, translational regulation of MGSA protein levels appears to be operative in that the MGSA protein is detected by immunocytochemistry only when serum factors are included in the culture medium. In a larger previously published immunocytochemical study of 32 other benign nevi cultured under serum-free conditions, immunoreactive MGSA protein was detected in only seven nevus cultures [3]. It has been previously demonstrated that, when growth factors are absent, the mRNAs for other proteins are not associated with polyribosomes and are no longer translated [25]. A similar mechanism may be operating for MGSA in nevocytes. Thus, during progression from melanocyte to nevocyte, there appears to be a deregulation of MGSA mRNA such that it is constitutively expressed, but the protein is only available when serum factors are provided exogenously. Subsequently, as progression to metastatic melanoma occurs, both MGSA mRNA and protein are constitutively produced regardless of availability of exogenous serum factors.

MGSA is only one member of a super family of β-thromboglobulin-related peptides that are structurally similar [21–25]. Several members of this family have been shown to be chemotactic for T-lymphocytes and neutrophils [26,27], suggesting that MGSA might also play a role in neutrophil/lymphocyte chemotaxis. Lymphocyte/neutrophil infiltration is thought to be an important indicator for the diagnosis of nevus atypia and possible tumor progression. Thus MGSA may be a key factor not only in the proliferation of melanocytes but also as a mediator of immune function.

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