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Growth Factor Modulation of Melanoma Growth Stimulatory Activity mRNA Expression in Human Malignant Melanoma Cells Correlates With Cell Growth

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This report demonstrates that the expression of melanoma growth stimulatory activity (MGSA) mRNA can be modulated in a positive fashion in the Hs294T human melanoma cell line by PDGF and MGSA. There is close correlation between MGSA expression and the pattern of cell growth in Hs294T cells.

Key words: MGSA/KC/gro, melanoma cells, expression modulation, cell proliferation

Cellular proliferation is controlled by external factors, among which the most important are hormones and growth factors (GFs) [1–3]. One of the most potent serum derived GFs identified is platelet-derived growth factor (PDGF) [4]. A variety of mesenchymal cells require PDGF for optimum growth in vitro [5, for review]. However, under growth restrictive conditions, PDGF by itself is not an efficient mitogen. A second set of GFs contained in the platelet-poor plasma fraction of the serum described as "progression factors" function synergistically with PDGF to promote the optimum mitogenic response [6,7].

PDGF treatment induces the mRNA of genes corresponding to the "PDGF competence family," including c-myc, c-fos, JE, JB, and KC [8,9]. Tumorigenic cells commonly have lower requirements for GFs than do the untransformed cells from which they are derived [10,11]. One cause of this decreased GF requirement is the production of "tumor-derived growth factors" by some transformed cells [12].

Melanoma growth stimulatory activity (MGSA) is an endogenous GF that is produced by human melanoma tumors and certain cultured nevus cells [13,14]. MGSA has been well characterized and purified from serum-free (SF) conditioned medium (CM) from the human melanoma cell line Hs294T [14–16]. Amino terminus sequence data have been obtained, MGSA cDNA clones have been isolated, and the nucleotide sequence of MGSA has been determined, demonstrating that the oligonucleotide sequence of MGSA is identical with the human gro gene [17] and is closely

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related to, if not identical with, the human KC gene (R. Derynck, personal communication). A cDNA probe for MGSA has been developed that can be used to evaluate MGSA mRNA expression in a variety of cell types [17]. In this report we describe the effects of MGSA and PDGF on expression of MGSA mRNA and cell growth in malignant melanoma cells.

MATERIALS AND METHODS

Cell Culture

The melanoma cell line Hs294T was cultured as previously described [14].

Source of Growth Factors and Other Materials

MGSA was obtained from the CM of Hs294T cells as described before [18]. PDGF was purchased from AMGen Biologicals, Thousand Oaks, California, as human, over 95% pure PDGF.

Experimental Design

Hs294T cells were transferred from T-150 stock flasks to 100 × 20-mm plastic petri dishes and kept on F-12 medium containing 10% fetal bovine serum (FBS) until they reached 60–70% confluency. The Hs294T cells were then partially synchronized by culturing on SF media for 24 hours. MGSA (0.3 ng/ml) was added to the SF culture media for different time periods, from 15 minutes to 24 hours. For other experiments, PDGF (2 ng/ml in SF), alone or in combination with MGSA (0.3 ng/ml) was added to the cells for 1 or 8 hours prior to extraction of RNA.

RNA Extraction

A 4 M solution of guanidine isothiocyanate was added directly to the monolayer cell culture or the cell pellet when a cell number assay was done. 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 3 M sodium acetate (NaAC) and ethanol precipitation [19].

Northern Blot Analysis

Twenty micrograms of total RNA was denatured at 65°C in the presence of formamide and formaldehyde and then subjected to electrophoresis on a 1.2% agarose gel in the presence of formaldehyde [32] and thereafter blotted to nitrocellulose filter. Hybridizations were performed using 50% formamide-containing solution, at 42°C, overnight. The 0.73-K/bp EcoRI fragment of the MGSA cDNA insert isolated from a human term placenta cDNA library was used as a hybridization probe [17], radiolabeled with [^{32}P] using a multiple DNA labeling system. Posthybridization washes were done at 42°C in 0.2 × SSC and 1% sodium dodecyl sulfate (SDS) to remove the probe in excess. Changes in the expression of MGSA mRNA were calculated by scintilligraphic analysis of the nitrocellulose membranes after hybridization with the [^{32}P]-labeled MGSA cDNA probe. The integrity of total-RNA was analysed by ethidium bromide stain of the samples. The position of the MGSA mRNA on the gel was determined by comparison with the position of the 28S and 18S bands of ribosomal RNA (r-RNA) in the stained gel.

Hs294T Cell Number Assay

Hs294T cells were transfered from T150 stock flasks to 100 × 20-mm plastic petri dishes (5×10^5 cells/dish) and incubated with F12 culture medium containing 10% FBS, to 60–70% confluency. The cells were then partially synchronized by culturing on SF medium for 24 hours. The corresponding additions of GFs were done at the concentrations mentioned before, and the cells were incubated for an additional 72 hours. On the 3rd day after GF additions, cell number was determined using a hemocytometer. The experiments were done by duplicate, and the results are expressed as absolute number of cells in millions (1×10^6).

RESULTS

Modulation of MGSA Expression by PDGF and/or MGSA

One hour after treatment of the Hs294T cells with 2 ng of purified PDGF, or 0.3 ng of MGSA per ml of SF medium, the expression of MGSA was not significantly enhanced when compared with the cells arrested on SF. However, MGSA mRNA expression was twofold enhanced by 8 hours treatment with either MGSA or PDGF. The use of PDGF combined with MGSA for 8 hours showed a synergistic enhancement in MGSA mRNA level (Fig. 1A).

Effects of PDGF and MGSA on the Growth of Hs294T Melanoma Cells

The effects of exogenously added PDGF (2 ng/ml), MGSA (0.3 ng/ml), or PDGF (2 ng/ml) plus MGSA (0.3 ng/ml) on the growth of Hs294T melanoma cells were evaluated in a 72-hour cell number assay. Addition of PDGF produced a 90% (±15) (1.4×10^6 cells) stimulation in cell proliferation when compared with Hs294T cultures mantained on SF media (0.658×10^6 cells). The addition of MGSA produced a 95% (±12) (1.44×10^6 cells) stimulation in cell proliferation with respect to the control culture (0.658×10^6 cells). The presence of both GFs simultaneously in the cell media produced a 128% (±18) (1.682×10^6 cells) stimulation in cell proliferation. (Fig. 2).

Modulation of MGSA Expression by MGSA

When the Hs294T cells were treated with 0.3 ng/ml of exogenous MGSA for different periods of time, from 15 minutes to 24 hours, the expression of MGSA mRNA was enhanced after 15–30 minutes, reaching a peak at about 2 hours after continuous exposure. MGSA mRNA expression began to decline after 4 hours of MGSA exposure, reaching undetectable levels after 24 hours (Fig. 3).

DISCUSSION

Polypeptide GFs regulate cell growth by enabling the cells to progress through the G1-phase of the cell cycle to reach the S-phase. To bring these events about, GFs must bind to specific receptors on the cell surface and induce a series of signals, which are relayed to the nucleus, where ultimately nuclear genes are induced and DNA replication ensues [22,23]. PDGF is a powerful mitogen released by platelets when blood clots [4,20]. PDGF induces in mesenchymal cells a stable state, termed "competence," by causing the activation or synthesis of cytoplasmic factors, which can be blocked by inhibitors of RNA synthesis [8]. PDGF by itself does not promote cell

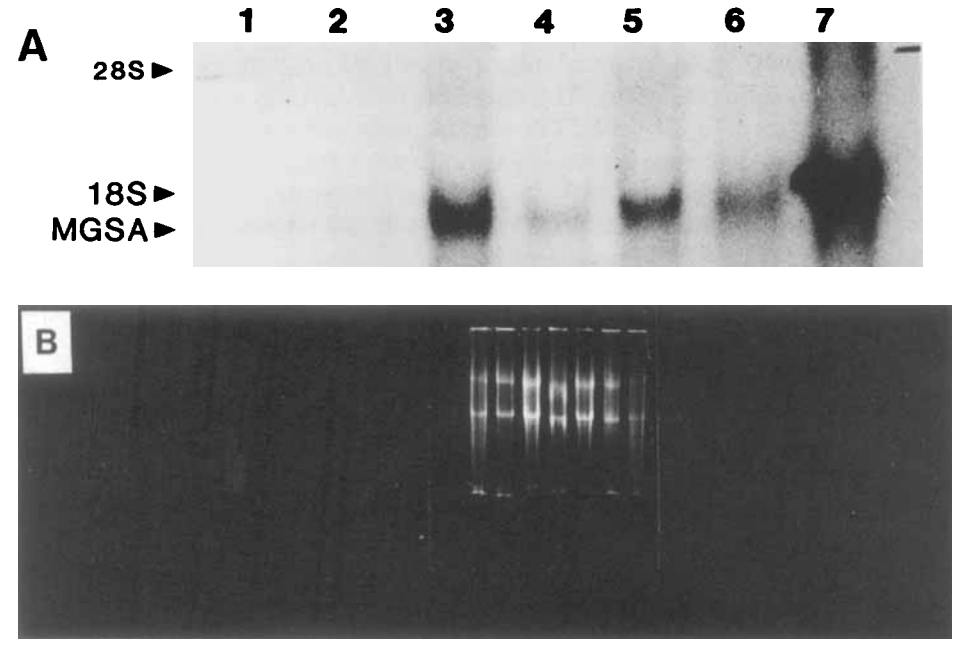

Fig. 1. MGSA mRNA detection by Northern hybridization of Hs294T melanoma cells treated with MGSA and/or PDGF. **A:** Cellular RNA was extracted by CsCl centrifugation and ethanol precipitation. Twenty micrograms of total RNA per sample was electrophoresed on a 1.2% agarose/formaldehyde gel. Levels of MGSA were determined by Northern blot analysis. The 0.73-kbp EcoRI fragment of the MGSA cDNA library was used as a hybridization probe. Hybridization was performed under high stringency conditions as described in Methods. The washed, hybridized membrane was exposed to a XAR-5 Kodak film for 48 hours to obtain the autoradiographic scan. MGSA mRNA levels in cells treated with serum-free medium (**lane 1**), MGSA (0.3 ng/ml) (**lanes 2,3**), PDGF (2 ng/ml) (**lanes 4,5**), or a combination of both (**lanes 6,7**), for 1 and 8 hours, respectively. The position of the 28S and 18S bands from the ethidium bromide stain of a duplicate gel (**B**) is indicated by the arrows. B: Ethidium bromide stain of total-RNA electrophoresed on agarose gel. A duplicate of the gel shown in A was electrophoresed and stained with ethidium bromide. The integrity of the RNA is shown by the 28S and 18S bands indicated by the arrows.

cycling, and the GFs present in platelet-poor plasma are required for cells to progress into S-phase [6,7]. Insulin-like GFs and EGF are two "progression" factors required for mouse fibroblasts to enter S-phase [24], after PDGF treatment. Several genes have been found to be responsive to PDGF stimulation in BALB/c-3T3 cells [8].

Of great interest here is the regulation of the MGSA/KC/gro gene, which is induced by PDGF and serum in fibroblasts [8], by EGF in endothelial cells and keratinocytes [25], by lectins in peripheral blood mononuclear cells [26], and by lipopolysaccharides (LPS) and colony-stimulating factor-1 (CSF-1) in macrophages [27,28]. The role of PDGF in the proliferation of melanoma cells is not clearly established [21]. Previous studies of [^{3}H]-thymidine incorporation into DNA of low-density Hs294T cultures have shown that PDGF lacked growth stimulatory activity for Hs294T cells under the conditions of the bioassay developed for MGSA [14]. However, the experiments reported here show that PDGF will promote increase in cell number in Hs294T cells in a 72-hour cell number assay. These results suggest not only

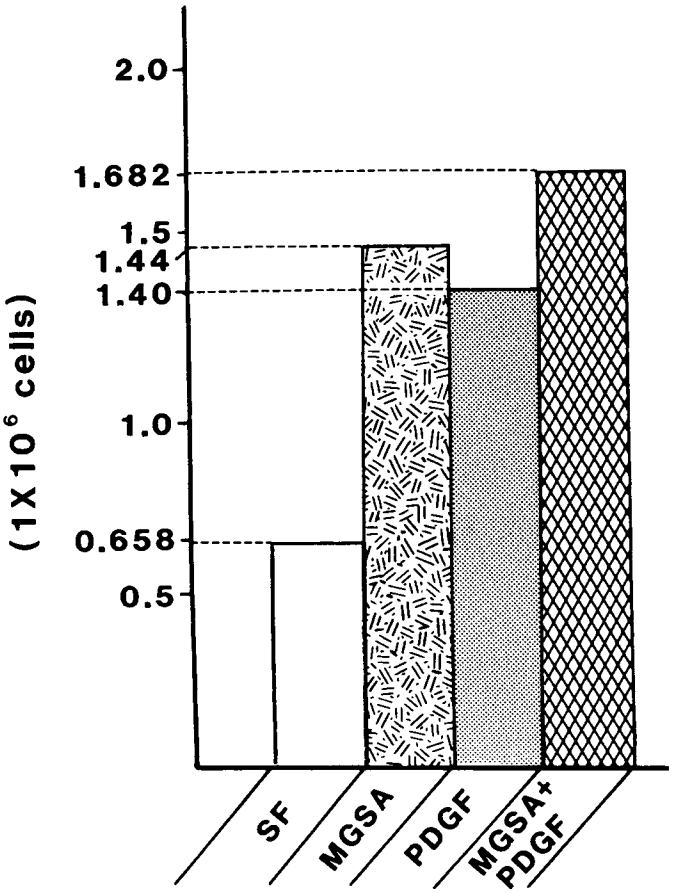

Fig. 2. Effects of MGSA, or PDGF, or both in combination on HS294T cell number: 5×10^5 Hs294T cells were plated in 100 × 20-mm plastic petri dishes and cultured to 60–70% cell density. After the cell cultures were partially synchronized by culturing on serum-free medium for 24 hours, Hs294T cells were treated with MGSA (0.3 ng/ml) alone or in combination with PDGF (2 ng/ml), in serum-free medium. After a 3-day incubation the medium was aspirated, the cells were released with trypsin, and cell counts were determined in a hemocytometer. Data are expressed as number of cells (1×10^6).

that there are functional PDGF receptors on Hs294T cells, but also that other endogenous GFs produced by the cells may be present in 72-hour Hs294T culture medium, which works in concert with PDGF to increase cell number. It appears that only suboptimal levels of these complementary endogenous growth factors are present in the 24-hour [^{3}H]-thymidine bioassay. In contrast, the increased incubation period and the greater cell density utilized in the cell number assay may provide the necessary levels of these complementary factors required for bioresponse to PDGF in Hs294T cells. IGF-I and MGSA are possible candidates for the other endogenous growth factors that are produced by Hs294T cells and work in concert with PDGF to promote Hs294T cell growth in SF culture medium during the 72-hour assay.

Other groups have reported that melanoma cells produce PDGF A-chain and to a lesser extent PDGF B-chain, but PDGF binding to PDGF receptors could not be

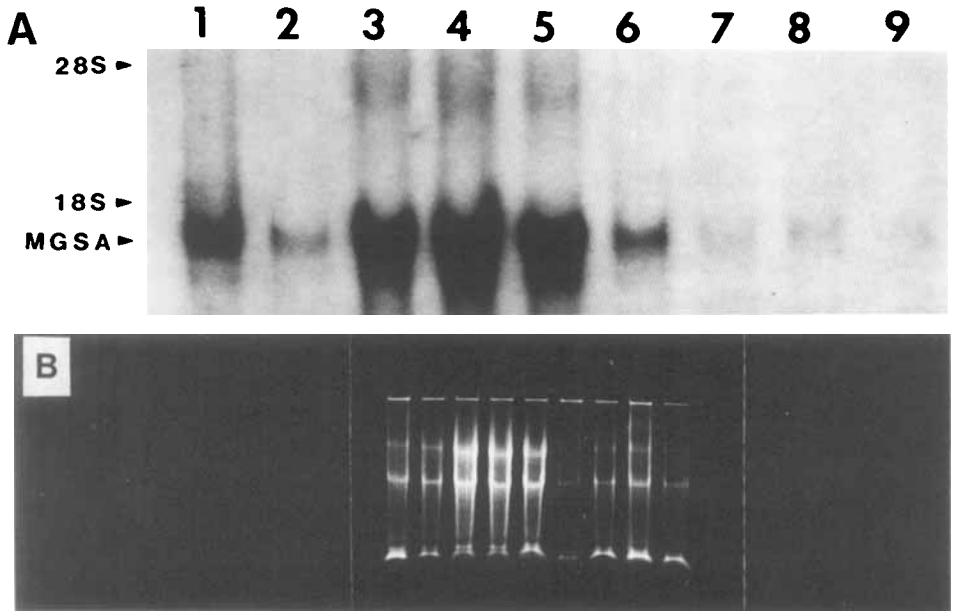

Fig. 3. MGSA mRNA detection by Northern hybridization of Hs294T melanoma cells treated with MGSA for different periods of time. **A:** Cellular RNA was extracted and processed as described in Figure 1A, and in "Materials and Methods." MGSA mRNA levels in subconfluent cell cultures kept on serum-free medium (**lane 1**), or treated with MGSA (0.3 ng/ml) for 15 or 30 minutes (**lanes 2 and 3**), or for 2, 4, 6, 8, 12, or 24 hours (**lanes 4–9,** respectively). The position of the 28S and 18S bands from the ethidium bromide stain of a duplicate gel (**B**) is indicated by the arrows. B: Ethidium bromide stain of total-RNA electrophoresed on agarose gel. A duplicate of the gel shown in A was electrophoresed and stained with ethidium bromide. The integrity of the RNA is shown by the 28S and 18S bands.

demonstrated in the melanoma cultures tested [31]. The Hs294T human melanoma cultures do contain a mRNA species, which hybridizes with the PDGF-R cDNA probe (G. Harsh and L.T. Williams, personal communication). Furthermore, antibodies that recognize the activated (tyrosine phosphorylated) form of the PDGF-R hybridize with a ~180-kD protein from serum-stimulated Hs294T cell extracts in Western blot analysis (G. Harsh, E. Balentien, and Lewis T. Williams, personal communication).

The significant stimulation of MGSA mRNA expression when the Hs294T cells are treated with PDGF suggests that one mode for PDGF induction of melanoma growth is through induction of MGSA mRNA, which is subsequently translated and secreted; MGSA would then bind to its receptor and exert a growth stimulatory effect on melanoma cells.

The pattern of MGSA message expression in the malignant melanoma cell line Hs294T is not significantly different from the profile observed in fibroblasts, where the early (1 hour) KC mRNA peak is followed by a rapid decline in the mRNA level at 4 hours [8].

Both PDGF, and TGF-alpha, have the ability to induce their own mRNA expression [29,30]. When Hs294T human melanoma cells were treated with exogenous MGSA, a significant increase of MGSA mRNA level was observed with a sig-

nificant induction at 2 hours, which dropped to basal levels after 6 hours of continuous exposure. The results imply the existence of an MGSA autoinduction, with MGSA being capable of amplifying the mitogenic response in melanoma cells. In the experiments reported here, there was variation in the timing of MGSA mRNA autoinduction. This variation is probably due to the inherent difficulty in synchronization of tumor cells at a specific point in the cell cycle. The enhanced expression of MGSA mRNA when the Hs294T cells were treated with MGSA correlated with a twofold MGSA stimulation of cell proliferation. In a previous report [17] we introduced cycloheximide (CHX) in the Hs294T cell culture to assess the mechanism of MGSA mRNA autostimulation. These experiments showed a superinduction in the message in response to MGSA when the cell culture was pretreated with CHX. These findings make it unlikely that de novo synthesis of proteins is involved in MGSA message autostimulation.

In this report we have shown that MGSA mRNA expression is induced in Hs294T melanoma cells by the addition of either exogenous MGSA or PDGF. This autoinduction is time dependent and correlates with cell growth. Further efforts will be directed toward the elucidation of the significance of MGSA mRNA in association with the complex molecular network leading to cell proliferation.

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