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Journal
Biochemical and biophysical research communications, 138(2)

ISSN
0006-291X

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Publication Date
1986-07-31

Peer reviewed

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Vol. 138, No. 2, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 31, 1986 Pages 795-802

BIOLOGICAL CHARACTERIZATION OF AN ACID-SENSITIVE GROWTH FACTOR FROM HUMAN PLATELETS: ROLE IN THE PROLIFERATION OF HUMAN MELANOMA AND BOVINE ENDOTHELIAL CELLS

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Received June 16, 1986

SUMMARY: An acid-sensitive growth factor for human melanoma has been partially purified from human platelets. TSK gel filtration HPLC provides a molecular weight estimation of 60,000 daltons. This factor is not only mitogenic for human melanoma, but also stimulates ^{3}H-thymidine uptake and increases the number of bovine aortic endothelial cells, while fibroblasts are nonresponsive. Radioiodination of active HPLC fraction has been accomplished. The human melanoma cell line, M1RW5 demonstrates specific, time-dependent binding of the labeled protein. These studies suggest that the growth of human melanoma may in part be regulated by a newly described growth factor present in human platelets. © 1986 Academic Press, Inc.

The control of normal cellular proliferation is mediated through the action of hormones and growth factors (1-3). Growth regulation of tumor cells is less well understood, although evidence suggests that tumor cells are capable of responding to growth signals as well as their normal counterparts (4,5).

A major storage site for many important biomolecules is the platelet. In addition to containing molecules essential for hemostasis (6), human platelets also store growth factors for a variety of cell types. PDGF induces the competent state in fibroblasts (7). TGF-B is a bifunctional growth regulator with

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Abbreviations: PDGF, platelet-derived growth factor; TGF-B, transforming growth factor beta; EGF, epidermal growth factor; MGF, melanoma growth factor; BES, N,N-bis[2-hydroxyethyl]-2-aminoethane sulfonic acid.

0006-291X/86 $1.50
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both inhibitory and stimulatory activities depending on cell type and culture conditions (8). An epidermal growth factor-like activity has been described in human platelets as well (9). EGF in combination with PDGF and TGF-B can induce the transformed phenotype in NRK fibroblasts. Another growth factor activity that has been demonstrated in human platelets, but is largely uncharacterized, which promotes growth of normal endothelial cells (10).

We have recently identified a novel melanoma growth factor activity from human platelets (11). This MGF is acid-sensitive and induces the expression of the transformed phenotype for melanoma cells in serum-free medium as measured by growth in soft agar. We have previously shown this activity to be distinct from PDGF, TGF-B, and EGF (11). We now describe the biological activity of a partially purified human platelet preparation on human melanoma and bovine aortic endothelial cells.

MATERIALS AND METHODS

Tissue cultures

The human melanoma cell line, M1RW5, was established from a patient tumor biopsy sample that had been successfully cloned in agar, as previously described (12). The cells were maintained in monolayer culture in DMEM (GIBCO, Santa Clara, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO), glutamine (0.8 ug/ml; Irvine Scientific, Santa Ana, CA).

The rodent fibroblast cell line, AKR-2B was provided by Dr. Harold Moses, Vanderbilt University, Nashville, TN. The bovine aortic endothelial cells were provided by Dr. John Castellot, Harvard Medical School, Cambridge, MA. AKR-2B cells were cultured in 10% FBS, DMEM, and the endothelial cells were cultured in 20% FBS, RMPI (GIBCO).

Gel filtration HPLC of platelet sonicate

The preparation of neutral platelet sonicate has been described (11). The crude sonicate was fractionated on a HPLC gel filtration column (TSK SW3000, Beckman Instruments, Berkeley, CA). The HPLC system used was Beckman Model System 334. Calcium and magnesium-free PBS, pH 7.5, was used as the eluent at a flow rate of 0.5 ml/hour. One milliliter fractions were collected. The fractions that were biologically active on M1RW5 cells were pooled, dialyzed against distilled water and lyophilized for further study.

Soft agar assays

Growth of human melanoma in soft agar has been described (11). The fibroblasts and endothelial cells were plated in soft agar in their respective maintenance medium. M1RW5 cells were plated at 5,000 cells/plate. The AKR 2-B cells were plated at 10,000 cells/plate. The endothelial cells were plated at 20,000 cells/plate. The active fraction from HPLC (1.75 ug/ml) was plated with each cell type in the overlayer.

Monolayer assays

The M1RW5, AKR 2B and endothelial cells were evaluated in monolayer culture for response to the active fractions from HPLC. The cells were seeded at a concentration of 300,000-500,000 cells/ 60 mm dish. After overnight incubation the assay medium was added with or without fractions from HPLC (43 ug/ml protein). M1RW5 assay medium was 1% BSA, DMEM. For all monolayer assays, the fibroblasts and endothelial cells were counted in 1% FBS, DMEM or RPMI. After 24-36 hours incubation at 37°C, the cells were harvested by scraping and counted using a hemocytometer counter.

The effect of fraction 16 on the incorporation of ^{3}H-thymidine (NEN, Boston, MA) into newly synthesized DNA was determined. 1-2 uCi/ml ^{3}H-thymidine was added to the dishes 18-24 hours after treatment with fractions from HPLC (43 ug/ml). Dishes were incubated at 37°C for one hour. Cells were harvested by scraping and washed three times with PBS with 100 ug/ml BSA. Trichloroacetic acid was added to the cell suspensions to achieve a final concentration of 10% TCA. The precipitate was collected and counted after overnight incubation at 4°C.

Binding Studies

Radiolabeling of fraction 16

Approximately 25 ug of protein from fraction 16 was radioiodinated with ^{125}I-labeled Bolton Hunter reagent (NEN; 13.4 mCi/ml). Lyophilized protein was dissolved in 50 ul of PBS, pH 8.0 and transferred to the reaction vial containing the reagent. The reaction was allowed to proceed at 4°C for 2 hours with occasional agitation. The reaction was stopped by the addition of 50 ul of 10 mM TRIS, PBS. Labeled protein was separated from free iodine by Sephadex G-25 chromatography using PBS as the eluent.

Time course of binding for M1RW5.

Confluent monolayers of M1RW5 cells in 12 well dishes were washed three times with binding buffer (DMEM, 1 mg/ml BSA, 50 mM BES, pH 6.8) to remove FBS. 400,000 cpm (650 ng) of labeled fraction 16 were added to each well in 0.5 ml of binding buffer. The dishes were incubated at room temperature for the times indicated. To determine nonspecific binding, a 500-fold excess of unlabeled fraction 16 was added per well prior to the addition of labeled protein. After the specified times, the assay was stopped by aspiration of the binding buffer. The cells were washed three times with cold buffer, and the cells were solubilized with 0.2 N NaOH for gamma counting.

Protein determination.

Protein concentrations were determined by using Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). This assay is based on the Bradford method utilizing Coomassie Brilliant Blue G-250 to assess protein binding.

RESULTS

Gel filtration HPLC of platelet sonicate

The fractionation of the crude platelet sonicate over the TSK SW3000 column resulted in 800-fold purification of the human melanoma stimulatory activity. Figure 1 shows the HPLC elution profile for the biological activity for M1RW5 melanoma cells. This molecular weight fraction was re-chromatographed through the SW3000 column and the melanoma stimulatory activity eluted in the same position, fraction 16. The molecular weight estimation from this column was 60,000. Active fraction 16s from this column were pooled, dialyzed and lyophilized.

Biological activity of fraction 16

The biological activity of HPLC fractions for human melanoma and bovine endothelial cells were compared to see if the activity

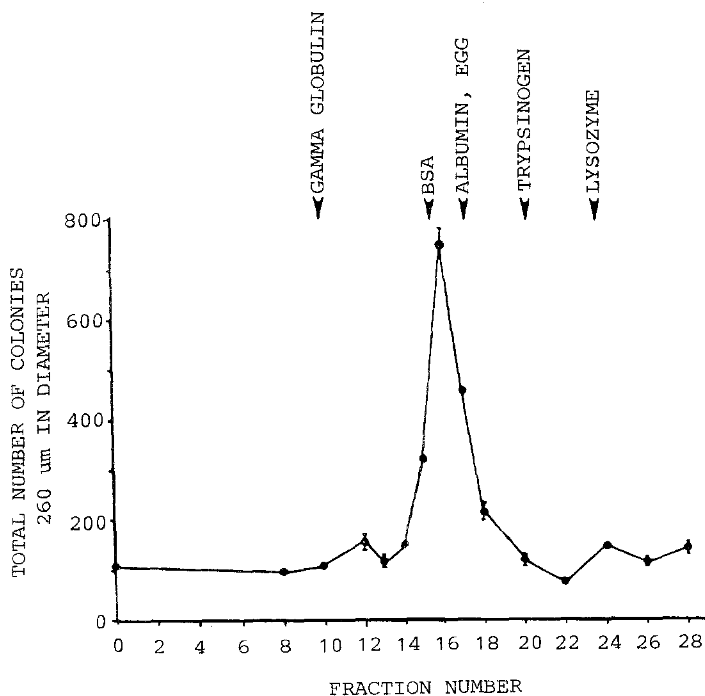

Figure 1. TSK gel filtration HPLC of human melanoma stimulatory activity. Molecular weight markers include: BSA (66,000); Albumin, egg (45,000); Trypsinogen (24,000); and Lysozyme (14,300). Gamma globulins mark the void volume.

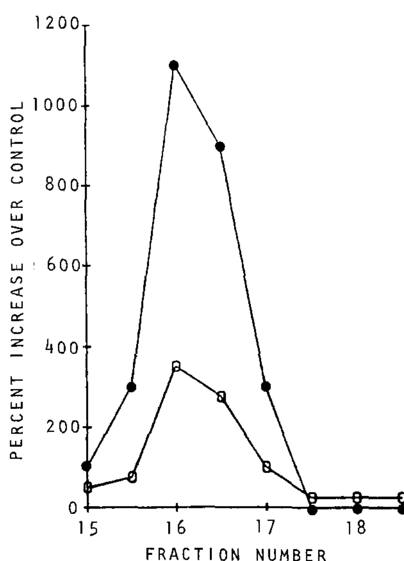

Figure 2. Soft agar responses of M1RW5 cells and monolayer responses of endothelial cells to TSK HPLC fractions. The M1RW5 cells (●) were plated in serum-free soft agar while the endothelial cells (□) were seeded in monolayer culture in medium containing 1% FBS.

co-purified. The TSK gel filtration fractions were assayed for mitogenicity on endothelial cell monolayer growth and melanoma soft agar growth. Figure 2 demonstrates that fraction 16 gave optimal stimulation of both cell types. To further assess the specificity of these responses, fibroblasts, melanoma and endothelial cells were evaluated for colony formation in soft agar and ^{3}H-thymidine uptake in monolayer. Table 1 summarizes the results of these studies. The M1RW5 cells were the only cells sensitive to signals that induced anchorage-independent growth; however, the endothelial cells were responsive to mitogenic signals for monolayer proliferation in addition to the melanoma cells. The fibroblasts were negative for mitogenic responses to fraction 16.

Figure 3 shows that the M1RW5 melanoma cells bind radiolabeled MGF from fraction 16 in a time-dependent manner. Binding begins as early as 10 minutes, maximizes within one hour and reaches a plateau which persists for at least four hours at

TABLE 1

Biological Activity of Fraction 16

	Bioassay		
	Soft agar growth (No. colonies) Control	Plus Fx 16	^{3}H-Thymidine uptake (% Control cpm)
Cell Type			
M1RW5	16+/-6	450+/-56	224 (p=0.004)
Endothelial	28+/-6	18+/-10	186 (p=0.08)
AKR 2B	22+/-8	32+/-8	106 (p=0.78)

P values are from student t test comparing control with fraction 16 treated cells.

room temperature. Seventy percent of binding was blocked by 500-fold excess cold fraction 16.

DISCUSSION

These studies demonstrate that human melanoma cells respond to a M_r 60,000 protein present in human platelets by induction of anchorage-independent and -dependent growth. In addition, the

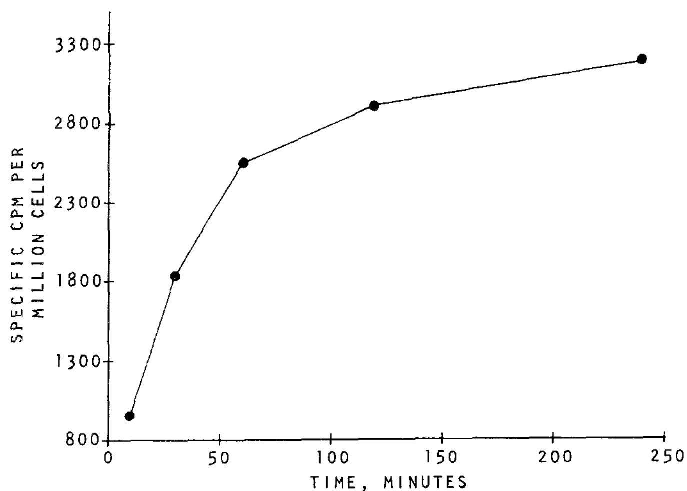

Figure 3. Time course of specific binding of MGF to M1RW5 cells. S.E. within triplicate determinations was less than 3%.

MGF stimulates anchorage-dependent proliferation of endothelial cells but not fibroblasts. This suggests that MGF may normally play a role in the regulation of endothelial cell growth. These results are in agreement with King et al.(10) who have identified an approximately M_r 65,000 endothelial cell growth factor in human platelets.

Furthermore, we have shown that human melanoma cells bind labeled MGF found in a HPLC-purified fraction of platelets. This preliminary study indicates that the melanoma cells possess specific receptors for MGF. We have previously shown that other growth factors present in platelets have no role in the growth regulation of human melanoma (11), and that this MGF is unique in its activity for melanoma cells. Purification of MGF will allow us to study the binding characteristics of this factor on the melanoma cells, as well as endothelial cells in order to address the role of this factor in the transformation process. The expression of receptors for MGF with subsequent sensitivity to it's mitogenic signals could potentially provide a growth advantage to melanoma cells during tumor formation and/or progression.

ACKNOWLEDGMENTS

The work was supported in part by grants from the National Cancer Institute (CA23704 and CA34689).

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