Production and Characterization of Mammary-Derived Growth Factor 1 in Mammary Epithelial Cell Lines†

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ABSTRACT: A mammary-derived growth factor, MDGF1, which stimulates collagen synthesis and proliferation in mammary epithelial cells was previously detected and purified from human milk and primary human breast tumors. MDGF1 binds to putative cell-surface receptors of 120–140 kDa and stimulates proliferation of normal and malignant human mammary epithelial cells. Partial protein sequence (N-terminal 18 amino acid sequence) shows that MDGF1 has no homology to any other known growth-promoting peptides. Polyclonal antiserum raised against this synthetic peptide recognizes native milk-derived MDGF1. We hypothesize that MDGF1 might be an autocrine or paracrine factor produced by and acting on normal and malignant human breast epithelial cells possessing MDGF1 receptors. As a first step in testing this possibility, we examined whether human breast epithelial cells in culture produce the growth factor. A protein with the size of MDGF1 was immunologically detected in the concentrated conditioned medium prepared from human breast cancer cell line MDA-MB 231, the mammary-derived but nontumorigenic HBL-100 line, and the normal reduction mammoplasty-derived, nonimmortalized 184 cell strain. A competitive radioreceptor assay (RRA) was used to estimate the level of MDGF1 in the conditioned medium. MDGF1 was present in the nanogram range per 1 million cells. A 62-kDa protein was detected in the above cell lysates by Western immunoblotting or by immunoprecipitation of metabolically labeled cell-conditioned media. The polyclonal antisera directed against the 18 amino acid peptide sequence from milk-derived MDGF1 could adsorb MDGF1 biological activity from conditioned medium. In vitro translation of cell mRNA yielded a protein of 55 kDa which was immunoprecipitated by anti-MDGF1 antibody. N-Glycosylation of MDGF1 was suggested by results of experiments using tunicamycin or *N*-glycanase treatment.

It was demonstrated earlier that synthesis of basement membrane collagen type IV is important for the growth and/or survival of the epithelium of the normal rodent mammary gland and of well-differentiated tumors derived from it (Kidwell et al., 1980, 1984; Wicha et al., 1979, 1980; Lewko et al., 1981). Our previous studies have shown that rodent mammary tumors that produce a basement membrane contain a growth factor that selectively amplifies the production of type IV collagen in cultures of mammary ducts and alveoli (Bano et al., 1983). The factor was not present in poorly differentiated rodent mammary tumors. We extended these studies to human breast cancer and human milk where we detected and purified a high molecular mass (62 kDa), acidic growth factor (p*I* = 4.8) named mammary-derived growth factor 1 (MDGF1)[1] from both sources (Bano et al., 1985; Kidwell et al., 1985). The factor can be distinguished from epidermal growth factor (EGF), which accounts for about 75% of the total growth activity in milk (Hollenberg, 1979), on the basis of size, p*I*, and disulfide bond reducing agent sensitivity. MDGF1 can also be distinguished from the three growth factors in milk described by Shing and Klagsbrun (1984a,b) and from colony-stimulating factor that Sinha and Yunis (1983) had detected in human milk.

MDGF1 stimulated cell proliferation and collagen synthesis of normal and malignant rodent and human mammary epithelial cells (Bano et al., 1985, 1990). It was demonstrated that MDGF1 binds to normal and malignant human breast epithelial cells ($K_D = 6 \times 10^{-9}$ M) (Bano et al., 1990) and A431 human epidermoid carcinoma membranes ($K_D = 2 \times 10^{-10}$ M) (Bano et al., 1985). All of these cells also increased their production of collagen in response to MDGF1 treatment, suggesting that the biological effects of the growth factor might be mediated through interaction with the putative membrane receptors (Bano et al., 1985, 1990). Our results also showed that biological effects of the growth factor are not seen with cell lines which had no detectable receptors for MDGF1 (Bano et al., 1990). Earlier observations also suggested that MDGF1 acts synergistically with estrogens since the growth factor does not stimulate in vitro mouse mammary cells depleted of estrogen in vivo by ovariectomy (Bano et al., 1985). On the basis of size, denaturation properties, and amino acid composition, we suggested that MDGF1 might be a new growth factor. This hypothesis has received further support by the current demonstration of a unique N-terminal amino acid sequence which distinguishes MDGF1 from any previously known mitogens (or other proteins).

The principal objective of the present study has been to raise polyclonal antibodies and to use the antisera to determine whether MDGF1 is synthesized by human breast cancer cells. Here, we demonstrate that a synthetic peptide, corresponding

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[1] Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diazonium salt; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IMEM, improved minimal essential medium; MDGF1, mammary-derived growth factor 1; NBT, nitro blue tetrazolium; NRK, normal rat kidney; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinylidene difluoride); SDS, sodium dodecyl sulfate; TGF, transforming growth factor.

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to the N-terminal 18 amino acids, can elicit high-titer antiserum in rabbits. Our results also indicate that MDGF1 is a secreted mitogen detected in the conditioned media of human MDA-MB 231 breast carcinoma and immortalized but nonmalignant HBL-100 cell lines, and the normal 184 mammary epithelial cell strain. Immunoprecipitations of N-glycosylated MDGF1 and products from in vitro translation of cellular mRNA indicated that MDGF1 is probably posttranscriptionally modified from a 55-kDa precursor.

Experimental Procedures

Cell Cultures. MDA-MB 231, MDA-MB 468, and HB-L-100 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were maintained in improved minimal essential medium (IMEM, Biofluids, Rockville, MD) in the presence of 5% fetal calf serum (FCS). The normal 184 cell strain and 184A1N4 (a carcinogen-immortalized subclone) were obtained from Dr. Martha Stampfer (Berkely, CA). 184 cells were maintained in a serum-free, pituitory extract, and growth factor supplemented MCDB 170 medium and 184 A1N4 cells were maintained in IMEM in 0.5% FCS in the presence of EGF (10 ng/mL, Collaborative Research), insulin (10 μg/mL, Sigma, St. Louis, MO), transferrin (10 μg/mL, Sigma), and hydrocortisone (0.5 μg/mL, Sigma) according to the published protocol (Stampfer & Bartley, 1985).

Preparation of Conditioned Medium. Cells were plated in T 175 cm^2 flasks and grown to confluence in IMEM supplemented with 5% fetal calf serum. Once confluent, the monolayers were switched to serum-free medium. After 24–48 h, the culture media were collected and concentrated 100-fold in an Amicon Ultra-filtration cell (YM 5 membrane, Amicon, Danvers, MA). The concentrated media were dialyzed against 100 volumes of distilled water at 4 °C. The material that precipitated during dialysis was removed by centrifugation. Protease inhibitors were added as described previously (Bates et al., 1986). The clarified sample was then lyophilized and stored.

Protein Sequencing. MDGF1 sequencing was accomplished by electroblotting onto PVDF [poly(vinylidene difluoride)] membranes (Immobilon Transfer, 0.45-μm pore size, Millipore) as described earlier (Matsudaira, 1987). Approximately 100 pmol of purified factor was loaded onto a 12% polyacrylamide minigel. After electrophoresis, electroblotting, and Coomassie Blue staining, the band corresponding to 62 kDa was cut out and used for amino acid sequencing in a gas-phase sequenator (Model 470A, Applied Biosystems, Foster City, CA). Sequence analysis was performed 3 times according to the protocol described by the manufacturer. Sequencing-grade reagents were used throughout. The analysis of primary amino acid sequence was performed on an IBM AT personal computer utilizing the "PC GENE" software (IntelliGenetics, Mountain View, Ca).

Predicted Location of the Major Antigenic Sites of MDGF1. The method of Hopp and Woods (1981) was used to determine an average hydrophilicity value for a series of adjacent amino acids. It is reported that there is a strong correlation between the highest average hydrophilicity value and an antigenic region of the molecule. The analysis was performed with an averaging group length of six amino acids which provide the most reliable predictions. The peptide was made using *t*-Boc chemistry on an automated peptide synthesizer (Bio Research, San Rafael, CA). Peptide synthesis reagents were purchased from Biosearch.

Antibodies. Antisera to MDGF1 were prepared by immunizing rabbits with the synthetic peptide corresponding to the N-terminal 18 amino acid residues. The immunogen was first conjugated to keyhole limpet hemocyanin (KLH) (Harlow & Lane, 1988) and was emulsified in complete Freund's adjuvant. Intradermal injections were given at multiple sites. Booster injections were given in incomplete Freund's adjuvant. Antibody titer was assayed by enzyme-linked immunosorbent assay (ELISA).

Measurement of MDGF1 Antibody Titer by ELISA. Microelisa plates (Dynatech-immunolon 11, Dynatech Laboratories, Inc, Chantilly, VA) were coated overnight at 37 °C with the growth factor (200 ng) or synthetic peptide (100 ng) in 100 μL of phosphate-buffered saline (PBS). The plates were washed 3 times with PBS containing 0.5% Tween-20 and blocked for 60 min with 1% BSA in PBS. The plates were washed again, and 100 μL of serial dilutions (1:200 to 1:25 600) of rabbit serum (preimmune and immune) in PBS containing 1% BSA was added for 120 min at 4 °C. After the plate was thoroughly washed with PBS–Tween, incubation was carried out for 1 h at 4 °C with goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Sigma, St. Louis, MO) at a dilution of 1:250/well. The plates were then washed 4 times with PBS–Tween and incubated with ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diazonium salt] in citric acid solution containing 0.012% hydrogen peroxide (Silman & Katehalski, 1966). The color absorbance was measured at 405 nm using a UR microplate Reader (MR 700 Reader, Dynatech Labs).

Radioreceptor Assay. Radioreceptor assay was performed by simultaneous incubation of test samples with ^{125}I-MDGF1 using membranes from A431 cells. About 3 μg of purified factor was iodinated using the Iodogen method (specific activity 62 μCi/μg) (Thorell & Johansson, 1971). Aliquots of membranes (2.5 μg of protein) were incubated with ^{125}I-MDGF1 (40 000 cpm/well) in the presence of increasing dilutions of conditioned medium in a total volume of 150 μL of binding buffer (IMEM with 50 mM HEPES, pH 7.4, containing 0.1% BSA). Following a 2-h incubation at room temperature, the wells were washed extensively and bottoms were cut and counted in a γ counter (Model B 5002, Packard Instrument Co.). Triplicate assays were done on each sample.

Preparation of Lysates and Western Immunoblotting. Cells were washed twice with PBS and then solubilized in SDS sample buffer (67 nM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol, and 0.3% bromophenol blue) at 100 °C for 5 min. Proteins in the cell lysates were loaded on the stacking gel (3.2%) and separated in the resolving gel with 12.5% acrylamide, under conditions of constant current and running at 30 mA per gel (Laemmli, 1970). The proteins were then transferred from SDS–PAGE onto 0.45-μm nitrocellulose filters (Schleicher & Schuell, Inc, Keene, NH) using electroblotting techniques (Towbin et al., 1979). The transfer buffer contained 150 mM glycine/20 mM Tris in 20% methanol, and the transfer was performed for 1 h at 150-mA constant current per gel at room temperature using a Hoefer trans-blot apparatus. After the transfer, filters were preincubated with 3% BSA in Tris-buffered saline containing Tween (TBST: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) overnight at 4 °C. A rabbit polyclonal antiserum raised against the synthetic peptide was used as the first antibody at a dilution of 1:250 or 1:500, and the blot is incubated for 2 h at room temperature. Goat anti-rabbit antiserum (IgG) linked to alkaline phosphatase (Promega) was used as the second antibody at a dilution of 1:7500, and the blot was incubated for 1 h at room temperature. The blots were then transferred to color-developing solution containing NBT (nitro

blue tetrazolium, 50 mg/mL, in 70% dimethylformamide) and BCIP (5-bromo-4-chloro-3-indolyl phosphate, 50 mg/mL, in dimethylformamide) in alkaline phosphatase buffer (Promega) (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$).

Pulse-Labeling with [^{35}S]Methionine and [^{35}S]Cysteine followed by Immunoprecipitation. Cells, growing in IMEM to 80% confluency, were washed twice with PBS and were incubated for 2 h at 37 °C in IMEM free of methionine and cysteine. The medium was replaced with fresh serum-free IMEM (lacking in methionine and cysteine), containing 200 μCi/mL [^{35}S]cysteine and [^{35}S]methionine (Amersham, Arlington Heights, IL, 1175 Ci/mmol). The labeled cells were collected and washed twice with PBS and lysed in 1 mL of radioimmunoprecipitation assay buffer (RIPA buffer: 100 mM Tris-HCl, pH 7.4, 300 mM NaCl, 2% Triton X-100, 2% sodiumdeoxycholate, 0.2% SDS, 0.4% BSA, and 2 mM PMSF). The lysate was cleared by centrifugation and stored at −70 °C. ^{35}S-Labeled proteins, released into the conditioned medium, were harvested after 16–24 h and clarified by dialysis and centrifugation. Immunoprecipitations of cell lysates or conditioned media were performed after incubation with antibodies (specific or nonspecific) for 2 h at 37 °C. Ten milligrams of inactivated *Staphylococcus aureus* was added, and the immunoprecipitates were washed extensively with RIPA buffer. Finally, the immunoprecipitates were solubilized in sample buffer at 100 °C for 10 min and were electrophoresed on denaturing polyacrylamide gels (12.5%). Gels were fixed with 10% acetic acid, treated with Enlightening (Dupont), dried, and exposed to X-ray film at −70 °C.

In Vitro Translation of mRNA and Immunoprecipitation. Total RNA from MDA-MB 231 and HBL-100 cells was subjected to in vitro translation using wheat germ extract. Approximately 10 μg of each RNA was translated for 90 min at 25 °C in a 50-μL reaction volume containing 100 μCi of [^{35}S]methionine (1200 Ci/mmol). The translated products were immunoprecipitated in the presence of specific and nonspecific antisera. The precipitates were analyzed by SDS–PAGE as described above and were detected by autoradiography.

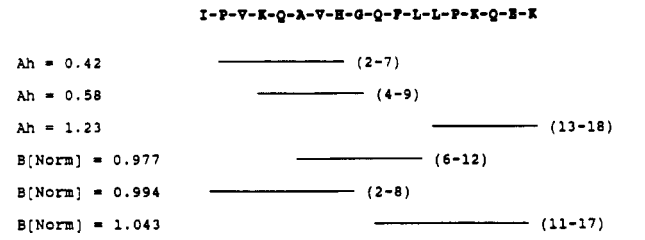
Tunicamycin Treatment. Tunicamycin (Sigma, St. Louis, MO) was prepared by dissolving in 50 mM sodium carbonate buffer (pH 10.0) and filter-sterilized with a 0.22-μm filter. Confluent monolayers of HBL-100 and MDA-MB 231 cells were incubated in IMEM in the presence or absence of 20 μg/mL tunicamycin (Bringman et al., 1987) for 4 h prior to metabolic labeling, which was performed as described above. Immunoprecipitation was carried out using MDGF1 antisera or prebled sera, and the immunoprecipitated products were run on 12.5% SDS gels as described earlier and autoradiographed.

N-Glycanase Digestion. About 0.5–1.0 μg of purified 62-kDa MDGF1 was dried in a Speed-Vac concentrator (Savant), redissolved in 0.5% SDS and 1.0% β-mercaptoethanol, diluted 5-fold into *N*-glycanase incubation buffer (0.2 M sodium phosphate, pH 8.6, and 1.25% Nonidet P-40), and treated with 10 units/mL *N*-glycanase (Boehringer Mannheim, West Germany) for 16 h at 37 °C. Samples were run on a 12.5% SDS–PAGE gel, subjected to Western blotting analysis, and silver-stained (Morissey, 1981).

Results

MDGF1 Partial Sequencing and Antibody Preparation. The N-terminal 18 amino acid sequence of MDGF1 was determined as indicated under Experimental Procedures. A search of the protein sequence data banks with the FASTP program revealed that MDGF1 was not closely related to any other growth factor. [TFASTA search revealed 35% homology (near the N-terminal region) to human pregnancy specific β_1-glycoprotein (HPSG) and about 41% homology to IL-1 β]. The 18 amino acid partial sequence was analyzed for hydrophilicity and flexibility to attempt to predict antigenic sites. As shown in Table I, residues 13–17 scored high for both parameters, indicating a high probability of an antigenic site in this region. A synthetic peptide corresponding to the sequence was prepared and coupled to KLH to be used as an antigen to raise MDGF1 antisera in rabbits. ELISA results indicated that the synthetic peptide generated antisera with moderately high titer which could be used for further assays.

Table I: N-Terminal Partial Amino Acid Sequence[a]



[a] Amino acid sequencing was carried out for the first 18 N-terminal residues of MDGF1. Using the Geneprobe program, the method of Hopp and Woods (1981) was used to predict average hydrophilicity (Ah) and chain flexibility (B[Norm]). Residues 13–17 represent overlap of the highest score for both parameters, indicating a high likelihood of an antigenic site localized here.

The factor purified from milk was subjected to SDS–PAGE and Western blotting using the rabbit polyclonal antisera at a dilution of 1:500. The antibody detected a major MDGF1 cross-reactive species as expected at 62 kDa (Figure 1A, lane 2). Approximately 100 ng of ^{125}I-MDGF1 was subjected to quantitative immunoprecipitations using MDGF1 antisera at two dilutions (1:100 and 1:250). More than 40% of MDGF1 was immunoprecipitated. As shown in Figure 1B, a major 62-kDa band was detected (lanes 3 and 4) which was not present when preimmune sera were used (lanes 1 and 2). The band diminished in the presence of increasing concentrations of unlabeled peptide (Figure 1C, lanes 6 and 7). Ten nanograms of unlabeled MDGF1 gave complete inhibition of the antibody reaction to ^{125}I-MDGF1.

MDGF1 in Normal and Malignant Breast Cell Lines. To determine whether various human mammary cell lines produce MDGF1, conditioned medium from the cell lines was analyzed by radioreceptor assay using the purified, labeled factor. In these experiments, normal 184 mammary epithelial cells, the nonmalignant HBL-100 breast cell line, and the highly malignant MDA-MB 231 hormone-independent lines were used. As shown in Table II, undiluted, 100-fold-concentrated medium conditioned by HBL-100, MDA-MB 231, and 184 showed maximum competition for the iodinated factor. The undiluted conditioned medium contained approximately 2–5 ng of MDGF1 receptor binding activity per 1 million cells. In contrast, the three other cell lines, MCF-7 (estrogen receptor positive), MDA-MB 468 (estrogen receptor negative), or 184A1N4 (carcinogen immortalized), did not show significant competing activity (Table II). In order to test whether the factor made by the above cell lines is biologically active, the conditioned media were subjected to partial purification on an isoelectric focusing column (IEF) (Bano et al., 1985), and the fractions with a *pI* of 4.8 were tested for biological activity. Normal rat kidney (NRK) cells were used as described previously (Bano et al., 1983, 1985, 1990), to determine

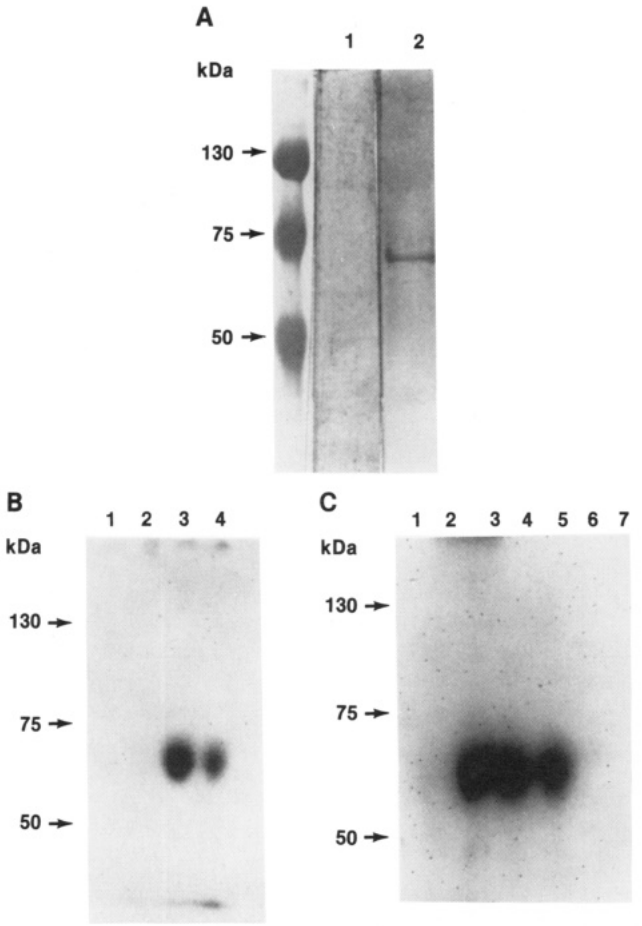


FIGURE 1: (A) Western blot analysis of MDGF1. About 20 ng of the purified factor from milk was run on a 10% SDS–PAGE and transferred to a nitrocellulose filter as described under Experimental Procedures. The blots were visualized following incubation with preimmune serum (lane 1) or with polyclonal antiserum to the synthetic peptide raised in rabbits at a dilution of 1:500 (lane 2). Molecular mass markers are indicated at the left in kilodaltons: phosphorylase *b*, 130; bovine serum albumin, 75; ovalbumin, 50. (B) Identification of the 62-kDa band by immunoprecipitation. ^{125}I-MDGF1 from milk (100 ng) was immunoprecipitated using MDGF1 antisera or preimmune sera at two dilutions. The samples were analyzed by SDS gel electrophoresis with a 10% gel and autoradiography. Lanes 1 and 2 represent samples treated with prebled sera and lanes 3 and 4 samples treated with anti-MDGF1 sera (1:100 and 1:250 dilutions). (C) Autoradiogram of a gel of immunoprecipitates of ^{125}I-MDGF1 from milk in the presence of unlabeled factor. Immunoprecipitation was carried out using anti-MDGF1 and prebled sera at a dilution of 1:100 as explained earlier. Unlabeled MDGF1 was used at various concentrations ranging from 0 ng (lane 3) to 2.5 ng (lane 4), 5 ng (lane 5), 10 ng (lane 6), and 25 ng (lane 7). Lanes 1 and 2 denote preimmune sera treated samples in the presence of 5 and 10 ng of unlabeled factor.

the optimal cell growth and collagen synthesis stimulation of the IEF fractions. The results shown in Table III suggest that the activity present in the conditioned media is biologically active in stimulating the growth of NRK cells and also in stimulating the synthesis of collagen. Purification by IEF and assay for collagen synthesis were carried out according to previously published methods (Bano et al., 1983, 1985). The antibody could be used to adsorb the biological activity of the factor present in the conditioned medium made by MDA-MB 231 and HBL-100 cells. The experiment was done by incubating purified factor or concentrated conditioned medium with anti-MDGF1 antisera either coated on wells or coupled to protein A–Sepharose beads. Then the growth factor or conditioned media were tested either in ELISA or for biological activity. As shown in Table IV, MDGF1 activity made by the conditioned medium was adsorbed out by the anti-MDGF1 antisera.

Table II: ^{125}I-MDGF1 Binding to A431 Membranes in the Presence of Unlabeled Cell-Conditioned Media[a]

additions	cpm bound	% competition
none	4660	
HBL-100	404	92
MDA-MB 231	392	93
MCF-7	3992	14
184	400	92
184A1N4	4045	13
MDGF1 (25 ng)	392	92

[a] Binding studies were performed with ^{125}I-MDGF1 (5×10^4 cpm). In all cases, the bound labeled MDGF1 represented less than 7% of the total ^{125}I-MDGF1 added. Data analysis was carried out in the presence of unlabeled growth factor or 100 μL of undiluted conditioned medium (concentrated 100-fold) normalized per 1 million cells for comparative purposes. The results represent the average of duplicate determinations.

Table III: Effect of Partially Purified Cell-Conditioned Media on Growth and Collagen Synthesis of NRK Cells[a]

IEF (pH 4.8) fractions added	stimulation (% control)		
	cell number	total cell protein	collagen
HBL-100			
10 μL	127 ± 6	155 ± 6	137 ± 5
25 μL	128 ± 3	177 ± 6	157 ± 6
50 μL	129 ± 4	183 ± 9	168 ± 6
100 μL	130 ± 2	161 ± 4	169 ± 8
MDA-MB 231			
10 μL	124 ± 4	129 ± 5	138 ± 3
25 μL	128 ± 4	150 ± 6	144 ± 5
50 μL	130 ± 2	167 ± 6	160 ± 4
100 μL	131 ± 3	178 ± 7	168 ± 5

[a] Conditioned media from HBL-100 and MDA-MB 231 cell lines were prepared as described under Experimental Procedures. The media were concentrated 100-fold and subjected to an isoelectric focusing column using ampholines of pH 3–10 range. The fractions eluting at pH 4.8 were collected and tested in NRK cell cultures for stimulation of cell growth and collagen synthesis, done according to the published methods (Bano et al., 1985). Other fractions from the IEF column eluting at pH 3.5 or 6.0 were tested as control, and they did not show any stimulatory effects. Values are shown relative to control (=100%). Mean ± SE of three experiments is given.

Table IV: MDGF1 Activity Adsorbed by Anti-MDGF1 Antiserum[a]

additions	ELISA absorbance 405 nm	at bioassay stimulation (% control)		
		cell growth	total cell protein	collagen
MDGF1 (10 ng)	1.900[b]	164 ± 3	162 ± 5	178 ± 4
MDGF1 (adsorbed)	0.212	102 ± 3	102 ± 2	102 ± 2
CM unadsorbed	1.279	145 ± 3	165 ± 3	161 ± 7
adsorbed on protein A (coupled to MDGF1 antisera)	0.468	109 ± 8	101 ± 1	102 ± 2
adsorbed on protein A (coupled to prebled)	1.568	146 ± 4	151 ± 7	167 ± 4

[a] Conditioned media (CM) from HBL-100 were concentrated 100-fold and used in the assays. A 10% suspension of protein A–Sepharose beads (200 μL in volume) was coupled to MDGF1 antisera (300 μg) or preimmune sera for 1 h at room temperature using constant rocking. Then the suspension is spun in a microfuge, and the supernatant is discarded. The pellet is then incubated overnight with the conditioned medium (300 μL) or 100 ng of MDGF1. After the mix was centrifuged, the supernatant was tested in ELISA and in bioassay performed as explained under Experimental Procedures. [b] Indicates color absorbance read at 405 nm after background subtraction using a microplate reader.

The polyclonal MDGF1 antibody was also used to detect the 62-kDa growth factor in cell lysates and conditioned media from the two human mammary cell lines HBL-100 and

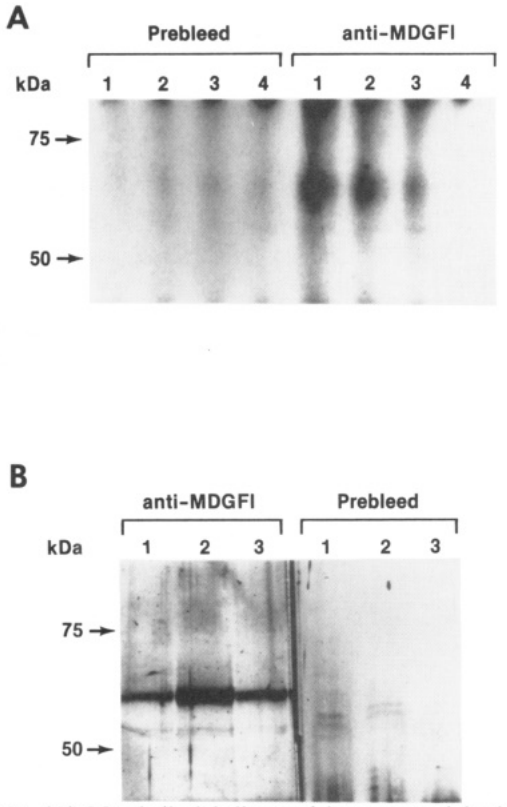


FIGURE 2: (A) Metabolic labeling and immunoprecipitations of conditioned media. HBL-100 cells were labeled with [^{35}S]methionine and [^{35}S]cysteine, and conditioned medium was immunoprecipitated either with prebled sera or with MDGF1 antisera at a dilution of 1:250. Unlabeled factor was added at concentrations of 0 (lane 1), 2.5 ng (lane 2), 5 ng (lane 3), and 10 ng (lane 4). The immunoprecipitates were resolved by electrophoresis on a 12.5% polyacrylamide gel and autoradiographed. (B) Western blot analysis of cell lysates from MDA-MB 231, HB-100, and 184 cells. About 30-μg samples of lysates from cells were run on a 12.5% gel, transferred to a nitrocellulose membrane, and processed. Lanes 1–3 denote lysates from MDA-MB 231, HBL-100, and 184 cells. The filters were treated with immune and preimmune sera, used at a dilution of 1:500.

MDA-MB 231. Metabolically labeled media from HBL-100 cell cultures were immunoprecipitated with antiserum as described earlier. Figure 2A shows the presence of a 62-kDa immunoreactive band (lanes 1 and 2) which disappeared in the presence of increasing concentrations of unlabeled factor (lanes 3 and 4). Ten nanograms per milliliter of unlabeled MDGF1 competed maximally for antibody reaction. Similar results were obtained with ^{35}S-labeled MDA-MB 231 conditioned media (figure not shown). Western blotting of cell lysates is depicted in Figure 2B. MDA-MB 231, HBL-100, and 184 cell lysates showed an immunoreactive band at 62 kDa. Immunoprecipitated product derived from in vitro translation of mRNA from the immortalized but nonmalignant HBL-100 cell line yielded a protein band with a molecular mass around 55 kDa (Figure 3A, lane 2). The same result was observed with MDA-MB 231 cell mRNA (figure not shown). In order to establish that MDGF1 might be a glycoprotein, tunicamycin treatment, metabolic labeling, and immunoprecipitation were done, and the result is illustrated in Figure 3B. Lane 2 depicts the 62-kDa band in the absence of tunicamycin, and lane 3 shows a lower molecular mass band (55 kDa) after tunicamycin treatment. Lanes 1 and 4 are immunoprecipitates with prebled sera in the presence (lane 4) or absence (lane 1) of tunicamycin. The result presented in Figure 3C demonstrates that treatment of purified growth factor with *N*-glycanase leads to a reduction in molecular mass, again to 55 kDa (lane 2), indicating that the mature 62-kDa

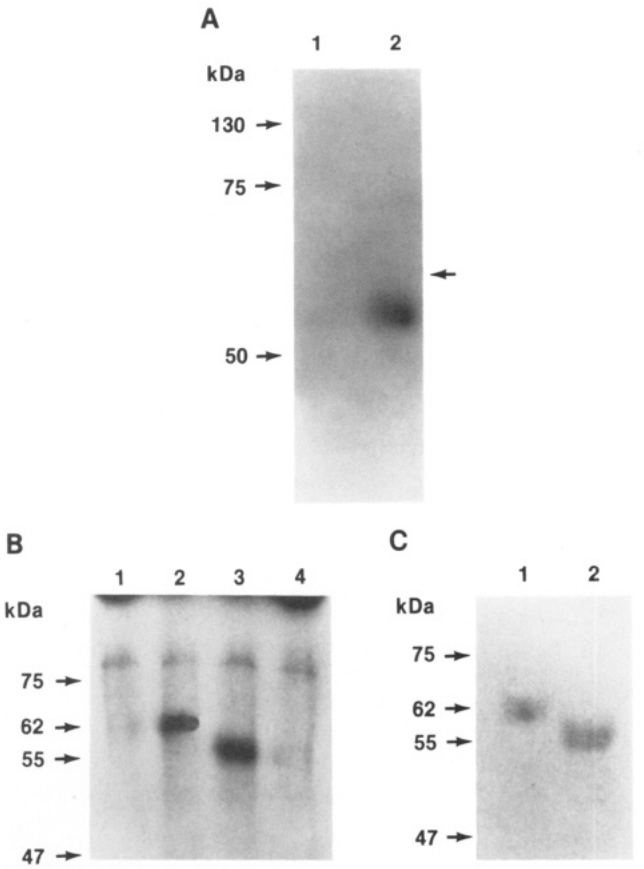


FIGURE 3: (A) In vitro translation of mRNA and immunoprecipitation. Total RNA was translated in vitro in a wheat germ system as described earlier and immunoprecipitated using MDGF1 antisera. The figure depicts the result for the HBL-100 cell line. Lane 1 denotes results with preimmune sera and lane 2 with immune sera, both used at a 1:250 dilution. The arrow at the right indicates the position of 62-kDa MDGF1. (B) Tunicamycin treatment. HBL-100 cells were grown to confluency and were treated with 20 μg/mL tunicamycin for 4 h at 37 °C. Metabolic labeling with [^{35}S]methionine and [^{35}S]cysteine was performed as described under Experimental Procedures. Samples of conditioned medium were immunoprecipitated using anti-MDGF1 or prebled sera at a dilution of 1:250. After solubilization, the immunoprecipitates were analyzed by 12.5% SDS–PAGE and subsequent fluorography. Lanes 3 and 4 denote tunicamycin treatment and lanes 1 and 2 in the absence of tunicamycin. Results for anti-MDGF1 sera are shown in lanes 2 and 3 and those for prebled sera in lanes 1 and 4. (C) N-Linked glycosylation of the 62-kDa MDGF1. Purified sample (0.5–1 μg) was incubated with 10 units/mL *N*-glycanase (lane 2) or buffer only (lane 1) for 16 h at 37 °C and subjected to Western blot analysis and silver-stained.

MDGF1 contains N-linked carbohydrates.

DISCUSSION

MDGF1, a Growth Factor of Unique Structure from Human Milk. Human milk is probably one of the richest sources of growth factors known (Klagsbrun, 1978, 1980; Klagsbrun & Shing, 1984; Sereni & Baserga, 1981; Shing & Klagsbrun, 1984a,b). The presence of growth factors in milk suggests that these activities may be produced by normal mammary epithelial cells. A series of other observations suggest that growth factors, such as TGF-α or EGF, may also perform normal physiological functions during the growth and development of the mammary gland during pregnancy (Vonderhaar, 1984; Salomon & Kidwell, 1988; Bates et al., 1988; Valverious et al., 1989; Zajchowski et al., 1988) and pathophysiologic functions in development of breast cancer (Bates et al., 1988; Valverius et al., 1989; Oka et al., 1988; Ennis et al., 1989; Artega et al., 1988). Growth factors could have autocrine,

Table V: MDGF1 Production and Properties by Various Cell Lines[a]

cell line tested	receptor binding	growth effects	production of MDGF1
MCF-7	++	++	-
MDA-MB468	++	+/-	-
184A1N4	++	++	-
184	+	++	++
MDA-MB231	-	-	++
HBL-100	+	++	++

[a] Radioreceptor assay and growth stimulation assays are done according to the protocols as described under Experimental Procedures. Binding studies showed high (++), moderate (+), and low (-) binding sites for MDGF1 on different cell lines (Bano et al., 1990). For growth assays, ++ denotes above 60% stimulation above control, and - denotes no stimulation. The biphasic growth effect is depicted by +/- which means growth stimulation at low concentrations and growth inhibition at high concentrations.

paracrine, and possibly even endocrine functions in proliferation of both normal and malignant breast tissue.

In our previous studies, we had detected an acidic growth factor (MDGF1) with an apparent molecular mass of 62 kDa. MDGF1 differentially stimulated the synthesis of collagen IV, and it binds to high-affinity binding sites on normal rat, mouse, and human mammary epithelial cells (Bano et al., 1985, 1990). In the current study, we now show that MDGF1 has an N-terminal 18 amino acid sequence unrelated to any other growth factor (or protein).

Antiserum Directed Against Biologically Active MDGF1: Detection in Milk and Breast Epithelial Cells. The N-terminal amino acid sequence of MDGF1 was obtained by cutting the 62-kDa band from the PVDF membrane and subjecting it to the sequencer. In a parallel experiment, a similar band was cut out from the gel, eluted, and tested for biological activity. These results and the fact that the polyclonal antiserum raised against the synthetic peptide recognizes 62-kDa MDGF1 give the indication that the sequence is derived from MDGF1. The polyclonal antibody was used to detect MDGF1 in human milk and breast cancer cell extracts by Western blot. The antibody could adsorb the biological activity of the factor as shown in Table IV.

Biosynthesis of MDGF1 by Breast Epithelial Cells. In vitro translation of mRNA from MDA-MB 231 and HBL-100 cell lines followed by immunoprecipitation using MDGF1 antiserum indicated the molecular mass of the putative precursor of MDGF1 to be slightly lower than that of the native protein (Figure 3A). This shift in the molecular mass might be due to the glycosylated nature of the factor. Experiments with tunicamycin (Figure 3B) and *N*-glycanase (Figure 3C) strongly suggest this possibility.

Normal mammary epithelial cells appear to possess the capacity to produce and respond to MDGF1. It is possible that MDGF1 is an autocrine growth factor for such cells. TGF-α has already been shown to be an autocrine growth factor for normal mammary epithelial cells and one breast cancer cell line in high-density culture (Bates et al., 1990; Ennis et al., 1989). At the present time, however, only one normal mammary epithelial strain in primary culture has been shown to both produce immunoreactive MDGF1 and respond to exogenous MDGF1 by proliferating. A possible autocrine loop cannot be proposed yet for other partially or completely transformed mammary epithelial cells. Table V summarizes the properties and production of MDGF1 by various cell types. Though MDGF1 is apparently not secreted by some responsive cell lines (like MCF-7 with moderate affinity binding sites) and some apparently nonresponsive lines secrete MDGF1 (like MDA-MB 231 which do not show detectable levels of MDGF1 receptors), we cannot yet rule out intracellular mechanisms of receptor/MDGF1 sequestration and autostimulation. Indeed, evidence has been presented for a mechanism of growth stimulation by growth factors synthesized and acting within a cell, in the absence of externalization and receptor binding (Derynck, 1987). MDGF1 might also serve a paracrine action in normal and/or malignant breast tissue since we have previously shown that it is a potent stimulator of collagen synthesis in fibroblasts (Bano et al., 1983, 1985).

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Identification of the Primer Binding Domain in Human Immunodeficiency Virus Reverse Transcriptase†

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ABSTRACT: We have labeled the primer binding domain of HIV1-RT with 5'-^{32}P-labeled $(dT)_{15}$ primer using ultraviolet light energy. The specificity of the primer cross-linking to HIV1-RT was demonstrated by competition experiments. Both synthetic and natural primers, e.g., $p(dA)_{15}$, $p(dC)_{15}$, and $tRNA^{Lys}$, inhibit $p(dT)_{15}$ binding and cross-linking to the enzyme. The observed binding and cross-linking of the primer to the enzyme were further shown to be functionally significant by the observation that $tRNA^{Lys}$ inhibits the polymerase activity on poly(rA)·$(dT)_{15}$ template-primer as well as the cross-linking of $p(dT)_{15}$ to the enzyme to a similar extent. At an enzyme to $p(dT)_{15}$ ratio of 1:3, about 15% of the enzyme can be cross-linked to the primer. To identify the domain cross-linked to $(dT)_{15}$, tryptic peptides were generated and purified by a combination of HPLC on a C-18 reverse-phase column and DEAE-Sephadex chromatography. A single peptide cross-linked to $p(dT)_{15}$ was identified. This peptide corresponded to amino acid residues 288-307 in the primary sequence of HIV1-RT as judged by amino acid composition and sequence analyses. Further, Leu(289)-Thr(290) and Leu(295)-Thr(296) of HIV1-RT appear to be the probable sites of cross-linking to the primer $p(dT)_{15}$.

Reverse transcriptases have been reported to utilize specific host tRNA for priming the viral first-strand DNA synthesis from the RNA template in vivo (Harada et al., 1975, 1979; Litvak et al., 1982). Avian myeloblastosis virus reverse transcriptase (AMV-RT)[1] uses $tRNA^{Trp}$ and MuLV-RT uses $tRNA^{Pro}$ while HIV1-RT utilizes $tRNA^{Lys}$ as a primer (Araya et al., 1980; Garret et al., 1984). The p66/51 heterodimeric form binds $tRNA^{Lys}$ in vitro with high affinity (Bordier et al., 1990; Barat et al., 1989, 1991). In vitro kinetic studies with synthetic homopolymers as template-primer have demonstrated

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[1] Abbreviations: HIV1-RT, human immunodeficiency virus 1 reverse transcriptase; AMV-RT, avian myeloblastosis virus reverse transcriptase; MuLV-RT, murine leukemia virus reverse transcriptase; dNTP, deoxynucleoside 5'-triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; DTT, dithiothreitol; NP-40, Nonidet P-40; TCA, trichloroacetic acid; TEAA, triethylammonium acetate.

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