A growth factor, mammary-derived growth factor 1 (MDGF1), has been purified to apparent homogeneity from human milk. The factor is a pepsin-sensitive, reducing agent-insensitive protein with a molecular mass of 62 kDa and a pI of 4.8. An apparently identical factor has been isolated from human mammary tumors, suggesting that MDGF1 might be made by and act as an autocrine growth factor for mammary cells. High affinity receptors for MDGF1 have been detected on mouse mammary cells, normal rat kidney cells, and A431 epidermoid cells (K_d = 2 × 10^{-10} M). MDGF1 at picomolar levels stimulates the growth of mammary cells and greatly amplifies their production of collagen, apparently via elevating collagen mRNA levels, an effect that is demonstrated for normal rat kidney cells.

The responsiveness of mammary cells to MDGF1 is attenuated when the cells are grown on a basement membrane collagen substratum, a component of the extracellular matrix upon which these cells normally rest in vivo. MDGF1 thus may regulate the production of new basement membrane as mammary epithelium invades the stroma during proliferation.

Basement membrane collagen (type IV collagen) synthesis appears important for the growth and/or survival of the epithelium of the normal rodent mammary gland and of well-differentiated tumors derived from it (1-4). In attempts to understand the factors regulating production of this protein, we discovered that mammary tumors that produce a basement membrane contain a growth factor that markedly and selectively amplifies the production of type IV collagen in cultures of mammary ducts and alveoli (5). Since poorly differentiated mammary tumors not producing a basement membrane lacked this growth factor, it was postulated that it might autoregulate basement membrane production. This mammary tumor-derived growth factor was highly purified and shown to be an acidic protein with a high molecular mass (68 kDa) (5).

Additional evidence for this postulate has been obtained. We have detected a similar type of growth factor in human milk and in human mammary tumors, as detailed in the present report. The latter activity, which is named MDGF1, has been highly purified. It is a 62-kDa acidic protein which electrophoreses on gels as a single band under denaturing conditions. A survey of the properties of known growth factors in human milk indicates that MDGF1 may be a new growth factor. It differs in size, pI, and disulfide bond reducing agent sensitivity from human epidermal growth factor (EGF) (6); it can be distinguished from the three growth factors in milk that Shing and Klagsbrun have described (7) on the basis of differences in size and/or pI and, on the basis of size, from a colony-stimulating factor that Sinha and Yunis (8) have detected in human milk.

MDGF1 binds to high affinity membrane receptors which are present on normal mouse mammary epithelium and on two cell lines that have been tested, namely A431 human epidermoid and normal rat kidney cells, which are fibroblasts. All of these cells also differentially increase their production of collagen in response to MDGF1 treatment, suggesting that the biological effects of the growth factor might be mediated through interaction with membrane receptors.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Normal mouse mammary ducts and alveoli were isolated from virgin female mice by collagenase digestion and differential filtration using the procedure of Wicha et al. (9) as modified by Kidwell et al. (10). The organoids were propagated in a serum-free medium (11) on tissue culture plastic dishes or on dishes coated with type IV or type I collagen (1). Cell number was quantitated following trypsin dissociation using a Biophysics particle counter (1). Normal rat kidney cells (NRK, clone F49) supplied from Dr. J. DeLarco, National Cancer Institute, were grown on tissue culture dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (5).

**Collagen Synthesis**—The procedure follows that outlined in Ref. 5. Briefly, assays were as follows. Cell cultures were grown for 2–3 days in the presence of [U-14C]proline (Amersham Corp., 280 mCi/mmol, 2 μCi/ml) or [3,4-3H]lysine (Amersham Corp., 85 Ci/mmol, 2–5 μCi/ml) with or without MDGF1. The growth medium was removed from the dishes, and the cells were scraped into 5 ml of 0.5 M Tris-HCl, pH 7.4, 0.11 M NaCl containing either unlabeled proline or lysine (1 mM). The cells were pelleted by centrifugation for 10 min at 1500 × g. After removing the supernatant, the pellets were resuspended in 50 mM Tris-HCl, pH 7.8, 1 mM proline or lysine and sonicated. Labeled protein was precipitated with 20% trichloroacetic acid containing 1 mM proline or lysine. The precipitates recovered by centrifugation were resuspended in 5% trichloroacetic acid and recentrifuged. This washing sequence was repeated three times. Precipitates were dissolved in 0.5 M NaOH. Aliquots were directly counted or analyzed for collagenase-sensitive radioactivity. In the latter case, 0.2 ml of solution was mixed with 0.1 ml of 1.0 Hepes buffer and 0.16 ml of 0.15 M HCl. Ten μl of 25 mM CaCl_2 and 0.02 ml of collagenase (Advanced Biofactures, form 111, 3200 units/ml of 0.05 M Tris-HCl, pH 7.6, 5 mM CaCl_2) were added. Samples were incubated for 90 min at 37°C. After cooling, 0.5 ml of 10% trichloroacetic acid was added, and the acid-insoluble material was centrifuged down at 4000 × g. Pellets were resuspended in trichloroacetic acid/tannic acid and recentrifuged. The combined supernatants (collagen) were counted. For control...
samples, the collagenase was replaced with collagenase buffer. In most cases, the differential stimulation of collagen synthesis by MDGF1 is presented. This was calculated by dividing the percent counts/min incorporated into collagen in MDGF1-supplemented cultures by the percent counts/min incorporated into collagen in cultures without MDGF1.

Laminin Assays—Laminin accumulation in mammary epithelial cell was estimated by enzyme-linked immunosassay using monospecific anti-laminin antibodies and purified laminin as a standard. Cells were cultured for 5 days in serum-free medium in the presence or absence of MDGF1. The medium was removed and the cell layer was washed with phosphate-buffered saline containing Tween 80 (12). Fifty μl of 1:100 diluted rabbit anti-mouse laminin antiserum or prebleed serum were added to the dishes which were incubated for 2 h at room temperature. Aliquots of this solution were added to microfiter wells coated with pure laminin, and the wells were incubated at 4 °C for 16 h. The wells were washed with phosphate-buffered saline/Tween 80, and peroxidase-conjugated goat anti-rabbit Ig antibody (Cappel Laboratories, 1:200 diluted) was added. Three h later, the dishes were washed with the same buffer, and the antibody bound peroxidase was quantitated using o-phenylenediamine and H₂O₂ as substrates (12).

Crude MDGF1 Preparation—Pooled milk (2 liters) from 5-10 donors was adjusted to pH 5.6 with acetic acid (13). The samples were centrifuged for 10 min at 15,000 × g at 4 °C. The floating lipids were removed and the supernatant fluid was recovered and dialyzed overnight against 100 volumes of 200 mM ammonium formate, pH 5.2, and then dialyzed over 100 volumes of 200 mM ammonium formate, pH 5.2. The fractions were lyophilized to dryness (∼3 mg/ml), and the insoluble residue was removed by centrifugation (5). The solution from each fraction was added and dialyzed against fresh 200 mM ammonium formate, pH 5.2. The samples were then chromatographed on a 1.25-cm TSK-3000 SW gel filtration HPLC column (Beckman). The proteins were eluted isocratically with the same buffer at a flow rate of 0.5-1.0 ml/min at 25 °C. One-ml fractions were collected, and 50-μl aliquots were screened for their effects on collagen synthesis in NRK cells cultures. Multiple runs were performed, and the peak fractions containing the collagen synthesis stimulating activity were pooled and concentrated by ultrafiltration or lyophilization. These samples were adjusted to 1 M NaCl in PBS and rechromatographed on the same column in 1 M NaCl/PBS. The peak fraction was then rechromatographed on the same column and again eluted with 1 M NaCl. Fractions of 1.0 ml were collected over the expected peak elution position and tested for collagen synthesis stimulating activity. These fractions, following reduction with β-mercaptoethanol (15), were run under nonreducing conditions on a 5% SDS slab gel. (3) After the series of purification steps, a single protein band was visualized on the gels stained with a silver reagent (18). Biological activity in this band was directly assessed by eluting the protein (without fixation or staining) from the slab gel (5) and assaying for its effects on mammary epithelial cell growth and collagen synthesis.

Amino Acid Analysis—Twenty-five μg of the purified proteins were hydrolyzed in constant boiling 6 N HCl for 18 h at 110 °C in a nitrogen atmosphere. The sample was decolorized with charcoal and evaporated to dryness in vacuo. A human serum albumin standard (Calbiochem-Behring) was similarly hydrolyzed to verify the analytical accuracy. Amino acids were separated by high performance liquid chromatography and post-column derivatized with ninhydrin for quantitation (17).

Radioceptor Assays—The purified MDGF1 was iodinated with ¹²⁵I using a modification of the method of Thorell and Johansson (18). Approximately 1 mCi of Na ¹²⁵I (Amersham Corp.) was mixed with 0.4 M sodium acetate at 0.05 μl of lactoperoxidase (Sigma, 0.8 mg/ml) were added followed by 5 μl of H₂O₂ (30% solution diluted 1:25,000). The H₂O₂ addition was repeated 15 s later, and after a second incubation, the reaction was terminated by adding a drop of saturated tyrosine (5 mg/ml). The fraction was then chromatographed on a 1.25 × 22-cm Sephadex G-25 column equilibrated in PBS containing 0.1% bovine serum albumin. The peak fraction was further purified by gel filtration HPLC (TSK-3000 SW) in PBS containing 1 mM NaCl. Receptor were quantitated on cell monolayers or with membranes prepared from A431 human epidermoid carcinoma cells according to Kinnab and Warren (19). In the latter case, aliquots of membranes containing 5 μg of membrane protein were incubated with 0.5-10 ng of labeled MDGF1 (specific activity 8 μCi/μg ± 500-fold excess of unlabeled MDGF1 in a total volume of 300 μl of 20 mM Hepes buffer, pH 7.4). Following a 30-min incubation at room temperature, the assay was terminated by adding a drop of saturated tyrosine (5 mg/ml). Approximately 1 mCi of Na ¹²⁵I (Amersham Corp., 410 Ci/mmol, 1 μCi/ml). After a 15-min incubation at 37 °C, the sample was cooled on ice and 2 μl of DNA polymerase I (Boehringer Mannheim, 0.1 μg/ml) were added. Incubation was performed for 45 min at 15 °C, and the reaction was terminated by the addition of 50 μl of 0.2 M EDTA containing 100 μg of calf thymus DNA/ml. Unincorporated radioactivity was removed by incubation with 10 mM Tris-Cl, 0.1 mM dithiothreitol, 0.1 mM MgCl₂, and bovine serum albumin (nuclease-free, Bethesda Research Laboratories, 1 mg/ml); 5 μl of DNAase I (Boehringer Mannheim, 0.1 μg/ml); and 2 μl each of the deoxyriphosphates 100 μg of calf thymus DNA/ml. Unincorporated radioactivity was removed by incubation with 10 mM Tris-Cl, pH 7.4, and 1 mM EDTA. Ten μg of salmon sperm DNA were added, and the DNA was then quickly digested with DNase I, 100 pg of calf thymus DNA/ml. Unincorporated radioactivity was removed by incubation with 10 mM Tris-Cl, pH 7.4, and 1 mM EDTA at 4 °C. Five μl of Nonidet P-40 (Shell Chemicals) were added, and lysis was performed for 5 min in the cold. An additional 5 μl of the detergent was added, and the suspension was centrifuged for 15 s at 15,000 rpm in a Beckman Microfuge. Fifty μl of the supernatant were mixed with 30 μl of 3 M NaCl, 0.3 M trisodium citrate, pH 6.8. Twenty μl of 37% formalin were added, and the sample was heated for 15 min at 60 °C. Samples were stored at −70 °C until ready for assay. Aliquots of this solution were applied to nitrocellulose filters, and the filters were baked and prehybridized exactly as described (21). Hybridization with the nick-translated probe was performed for 20 h under stringent conditions, and the filters were washed as described (22). Autoradiograms were developed for 24 h using RPI film from Agfa-Gevaert. The α(1R1) cDNA against type I collagen was kindly supplied by D. Rowe, University of Connecticut (23). In some cases, RNA blot hybridization was performed. In this case, RNA was prepared with guanidinium thiocyanate following published procedures (23).

RESULTS

Purification and Properties of MDGF1—To determine whether a collagen synthesis stimulating activity was present in human breast tumors and human milk, which is known to contain a variety of hormones and growth factors (24), sam-
samples of delipidated, casein-free human milk or acid/ethanol extracts of tumors were subjected to isoelectric focusing. The fractions obtained were analyzed for collagen synthesis stimulating activity using NRK cells (Fig. 1). In both preparations, there was a major peak of collagen synthesis stimulating activity which focused with a pI of 4.8. In the assay, the diazoylated, lyophilized fractions were tested for the ability to stimulate labeled amino acid incorporation into collagenase hydrolyzable protein in NRK cells. In this analytical assay (Fig. 1), 15 ml of milk and 25 mg of tumor protein extract were applied to the isoelectric focusing column. No significant effect of the fractions on total, non-collagen protein labeling was observed, indicating a differential stimulation of net collagen synthesis by 8- and 6-fold with the tumor and milk preparations, respectively.

Preparative isoelectric focusing runs were then performed with human milk. Fractions with the appropriate pI (4.8) were combined for further purification. Milk was initially chosen as a preferred starting material owing to the large amounts of material which could be obtained. For each isoelectric focusing run, 100-200 mg of milk protein were routinely processed. Pooled fractions were dialyzed against ammonium formate and then lyophilized to dryness. Residues were dissolved in minimum volumes of 0.15 M NaCl and 0.01 M NaH2PO4, pH 7.4, and the samples were chromatographed on a preparative HPLC TSK-3000 SW gel filtration column in the same buffer. Aliquots of the column fractions were directly tested for their effects on collagen synthesis using NRK cells. As shown in Fig. 2, a major and minor peak of collagen synthesis stimulating activity was detected with apparent molecular weights of ~60,000 and less than 10,000, respectively. The high molecular weight activity represented less than 5% of the total protein applied to the column as estimated from the A280 profile of the fractions (Fig. 2). Radioreceptor assays of the fractions indicated that the small molecular weight species was human epidermal growth factor since there was potent competition with 125I-EGF for binding to A431 cell membranes in the presence of this fraction (data not shown). The high molecular weight species was further purified as described below.

Fractions from multiple HPLC runs exhibiting an elution time of 11.5-12.5 min (Fig. 2) were pooled, dialyzed against ammonium formate, lyophilized, and then re-chromatographed on the TSK-3000 SW column using phosphate-buffed saline containing 1 M NaCl as the eluting buffer. The fractions eluting between 11.5 and 12.5 min from this step were re-chromatographed on the same column in the high salt buffer. One-ml fractions were collected between 11 and 15 min. These were dialyzed against water, lyophilized to dryness, reuspended in PBS, and assayed for collagen synthesis stimulating activity using NRK cells or analyzed by SDS-gel electrophoresis following reduction and denaturation. The results presented in Fig. 3 demonstrate that a single protein band of approximately M_r = 62,000 was present in the fraction eluting from the TSK-3000 SW column at 12 min. All of the biological activity was confined to this fraction.

Purification of MDGF1 was monitored by assessing the extent of differential stimulation collagen synthesis in NRK cells. Using this assay, an estimate of the purification was made. Optimal stimulation with crude decaesinate, delipidated milk required 250 μg of protein/ml of medium in the NRK cell cultures. After the isoelectric focusing step, this value was reduced to 35 μg/ml, while after the first HPLC TSK-3000 SW column run, the value was about 0.25 μg/ml. Following the final gel filtration step on the TSK-3000 SW column (1 M NaCl buffer), a maximal response was observed at 10 ng of MDGF1/ml (Fig. 4), indicating an overall purification of 2500-fold.

**Fig. 1. Fractionation of MDGF1 by isoelectric focusing.** Tumor extracts or milk preparations were focused as described. Three-ml fractions were recovered and dialyzed against PBS, and then 50-μl aliquots were added to NRK cell cultures. Following a 3-day cultivation in the presence of 2 μCi/ml [14C]proline, the cell layers were harvested. The presence of radioactivity converted from trichloroacetic acid-insoluble to an acid-soluble form by protease-free collagenase digestion was tested. ◦, per cent total counts/minute in collagen; ◻, pH, Tsp, human tumor extract; □, pH, Tsp, human milk. The arrows denote the focusing position of mouse EGF.

**Fig. 2. HPLC chromatography of MDGF1.** The pH 4.8 fraction from the isoelectric focusing step was chromatographed on a TSK-3000 SW column with PBS as the eluting buffer. Fifty-μl aliquots of each fraction were assayed for effects on collagen synthesis. The results (fold differential stimulation) were calculated by dividing the percent counts/minute in collagen of treated cells by percent counts/minute in collagen of cells cultivated with 50 μl of PBS. A value of 1 indicates no differential stimulation of collagen synthesis. V_e, void volume. A, elution position of serum albumin standard; B, elution position of mouse EGF standard. ◦, collagen synthesis; - - - , A_280.
Mammary-derived Growth Factor

**Fig. 3.** HPLC gel permeation chromatography of MDGF1 using high ionic strength eluting buffer. Fraction 12, obtained from several HPLC runs under low salt conditions, was pooled and re-chromatographed on the same column using 1 M NaCl/PBS as the eluting buffer. Fractions were tested for effects on collagen synthesis (graph) and analyzed by SDS-gel electrophoresis after reduction with β-mercaptoethanol. Inset A, analysis of fractions 11–13 on 7.5% gels; inset B, analysis of fraction 12, the biologically active fraction, on 15% gels. The gels were fixed and stained with a silver reagent as described under “Experimental Procedures.”

**Fig. 4.** MDGF1 effects on collagen synthesis in NRK cells. NRK cells were incubated with MDGF1 at the indicated concentrations, and the differential effects on collagen synthesis were determined as described in the legend of Fig. 2. Cells were grown on tissue culture plastic dishes.

**Fig. 5.** Comparison of tumor and milk MDGF1 species by SDS-gel electrophoresis. The two species were purified by isoelectric focusing and HPLC chromatography under low and high ionic strength conditions. Samples were reduced with β-mercaptoethanol before electrophoresis. Lane A, molecular weight standards; lane B, MDGF1 from milk; lane C, MDGF1 from human mammary tumors. Five μg of protein/lane for MDGF1 from both sources were used.

Fractionation was at least 25,000-fold. When cell counts were performed on NRK cells grown in absence or presence of purified milk MDGF1 (25 ng/ml) for 3 days, no effect of the factor on NRK cell number was observed (8.4 × 10⁶ cells/dish in the absence or presence of MDGF1).

An activity present in human tumor extracts apparently identical to MDGF1 was also purified using the methods outlined for the milk factor. The electrophoretic property of the purified preparation was compared to MDGF1 obtained from human milk (Fig. 5). As indicated, a single silver stained band (Mₐ = 62,000) was present in both the tumor and milk samples when analyzed on 7.5% SDS slab gels under denaturing and reducing conditions. Starting with 5 liters of milk (15 g of protein), approximately 115 μg of MDGF1 were recovered, while approximately 80 μg of the tumor factor were recovered from 100 g of the human tumor (wet weight). The specific activities of the milk- and tumor-derived factors were about equal in a collagen synthesis assay with NRK cells (not shown).

To ascertain that biological activity was present in the protein band on the gel, 5 μg of MDGF1 from human milk were electrophoresed along with a fluorescent serum albumin marker. Slices of gel just below the fluorescent marker (localized by UV) were cut from the gel and eluted in PBS/bovine serum albumin. Control gel slice extracts were also prepared from the same gel regions. These fractions were analyzed for their effects on collagen synthesis in mammary cell cultures. The results presented in Table I demonstrate that biological
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Recovery of biological activity in the 62-kDa band following gel electrophoresis

Five μg of MDGF1 were electrophoresed on 7.5% gels. Gel areas corresponding to the MDGF1 band or control gel lanes were cut out and solubilized in 6 ml of PBS/bovine serum albumin for 48 h at 4 °C. The solubilized material was recovered by centrifugation and filter-sterilized. Aliquots were added to cultures of mammary ducts and alveoli along with [3H]lysine (5 μCi/ml). Following 3 days of incubation, the amount of incorporation into total protein and into collagen was measured.

| Addition                  | Total protein (cpm × 10^-4) | Collagen (cpm) | In collagen (% cpmp) |
|---------------------------|----------------------------|----------------|---------------------|
| Control gel extract, 50 μl| 0.7                        | 130            | 0.02                |
| Control gel extract, 75 μl| 0.8                        | 70             | 0.01                |
| MDGF1 gel extract, 50 μl  | 0.9                        | 590            | 0.07                |
| MDGF1 gel extract, 75 μl  | 1.1                        | 470            | 0.04                |

Table II

Chemical stability of MDGF1

The factor was treated according to the protocols described and tested for its effects on collagen synthesis in NRK cell cultures. The fold differential stimulation was calculated as described for Fig. 2. Twenty-five ng of MDGF1 were added per ml of growth medium in each case.

| Addition                                | Collagen synthesis | -fold differential stimulation |
|-----------------------------------------|--------------------|--------------------------------|
| MDGF1                                   |                    |                                |
| Untreated in PBS                        | 3.4                |                                |
| β-Mercaptoethanol-reduced                | 3.0                |                                |
| Heated at 90 °C                         | 2.9                |                                |
| Pepsin digested                         | 1.1                |                                |
| β-Mercaptoethanol buffer                 | 0.9                |                                |
| Pepsin digestion buffer                  | 1.3                |                                |
| PBS                                     | 1.0                |                                |

Effect of Disulfide Reduction and Heating—One hundred ng of MDGF1 from milk were preincubated in the absence or presence of 40 mM β-mercaptoethanol for 2 h at 25 °C in 1 ml of PBS, pH 7.4. MDGF1 was then added to NRK cells, and the incorporation of [3H]proline into collagen was assessed after 3 days. The reducing agent treatment had no appreciable effect on the potency of MDGF1 as shown in Table II. There was a 340% differential stimulation of collagen synthesis using untreated MDGF1 and a 100% differential increase with MDGF1 treated with β-mercaptoethanol.

In a similar experiment, 100 ng of MDGF1 in PBS were heated for 10 min at 90 °C followed by bioassay. The factor was found to be essentially unaffected by this treatment as shown in Table II.

Protease Sensitivity of MDGF1—To determine whether MDGF1 was protease-sensitive, 100 ng of MDGF1 from milk were digested with pepsin (100 ng) in 1% acetic acid for 1 h at 37 °C. Controls including pepsin only and growth factor without pepsin but incubated in acetic acid were also included. Following incubation, all the samples were neutralized and tested for their effects on collagen synthesis. As shown in Table II, the biological activity of MDGF1 was found to be completely destroyed by pepsin digestion.

Amino Acid Composition—The amino acid composition of milk MDGF1 was determined on acid-hydrolyzed samples. As anticipated on the basis of pI, there was an excess of acidic over basic amino acids (Table III).

Cell Membrane Receptors—To determine whether milk MDGF1 might be acting via specific membrane receptors, purified milk MDGF1 was iodinated and radioreceptor assays were performed on intact NRK and mouse mammary cells (11) or with human A431 epidermal carcinoma cell membranes (19). Specific binding was detected in all cases with approximate saturation of binding with A431 cell membrane occurring at 10 ng/ml (Fig. 6, inset). A Scatchard analysis using the membrane preparation is depicted in Fig. 6. The affinity constant was calculated to be 2 × 10^-10 M with 1.33 pmol of milk MDGF1 specifically bound per mg of membrane protein. MDGF1 binding appears to be specific since the inclusion of 2.5 μg of EGF in the radioreceptor assay failed to inhibit the binding of 125I-MDGF1 (5 ng). Likewise, 2.5 μg of MDGF1 did not affect the binding of 125I-EGF (2.5 ng) to EGF receptors on these membranes (data not shown).

Mechanism of MDGF1 Action—The effect of milk MDGF1 on collagen production by NRK cells was due to an amplification of collagen synthesis rather than to a decrease in

Table III

Amino acid composition of MDGF1

Acid hydrolysates were prepared as described. A human serum albumin standard was prepared for assay in an identical manner, and corrections for losses were made using the known composition of the standard. The molar ratio was calculated from the number of picomoles of amino acid divided by the number of picomoles of histidine.

| Amino acid                     | Molar ratio |
|--------------------------------|-------------|
| Aspartic acid + asparagine     | 3.7         |
| Threonine                      | 2.0         |
| Serine                         | 4.1         |
| Glutamic acid + glutamine      | 7.7         |
| Glycine                        | 5.8         |
| Alanine                        | 2.3         |
| Valine                         | 2.6         |
| Methionine                     | 2.2         |
| Isoleucine                     | 2.2         |
| Leucine                        | 4.4         |
| Tyrosine                       | 1.8         |
| Histidine                      | 1.0         |
| Lysine                         | 2.4         |
| Arginine                       | 1.0         |
| Proline                        | 2.1         |
| Cysteine                       |             |

Fig. 6. MDGF1 receptor analysis. 125I-MDGF1 binding to A431 cell membranes is shown. Graph, Scatchard analysis; inset, total binding (○) and specific binding (●).
collagen turnover, as illustrated by pulse-chase studies shown in Fig. 7. Milk MDGF1 (100 ng/ml) or control buffer was added to cells with a labeled precursor amino acid. After 15 min of labeling, either the cells were harvested or the medium was changed to remove the unincorporated amino acid, and the cells were grown an additional 24 h. The amount of labeled collagen associated with the cells was then measured using the collagenase assay method. Within 15 min, MDGF1 differentially stimulated collagen labeling by approximately 3-fold. There was little difference in collagen turnover following a 24-h chase period whether MDGF1 was present or not.

The abundance of collagen mRNA-related sequences in NRK cells treated with and without the milk-derived growth factor was then assessed using cDNA probes against type I collagen, the major collagen species made by these cells. 3 Cytoblot analyses using the α1(R1) probe indicated a difference of approximately 4-fold in the amount of collagen I mRNA-related sequences detected in cells after 2 days of culture with MDGF1 (Fig. 8). A similar dot blot hybridization assay was also performed with 15 μg of total cellular RNA with comparable results (data not shown). Both assays were performed under stringent hybridization conditions.

Effect of MDGF1 on Mammary Epithelial Cells—In contrast to NRK cells, mouse mammary duct and alveolar cells treated with milk MDGF1 exhibited an increased rate of growth. MDGF1 also enhanced the level of collagen production. The dose-response relationship between growth stimulation or collagen synthesis effects and MDGF1 concentration is shown in Table IV. Optimal stimulation of both cell division and collagen synthesis was produced at or below a MDGF1 concentration of 5 ng/ml.

The effect of MDGF1 was tested on collagen synthesis in mouse mammary ductal and alveolar cells propagated on different substrata, a condition which has been previously demonstrated to influence the responsiveness of these cells to other growth factors. Fig. 9 demonstrates that when cells were plated on type IV collagen-coated dishes there was no effect of MDGF1, whereas cells which had been plated on type I collagen-coated dishes or on tissue culture plastic dishes differentially increased their production of collagen by 3- and 4-fold, respectively, in response to MDGF1. This difference in responsiveness could not be accounted for by a differential absorption of the MDGF1 to any of the types of culture substratum as shown by incubating medium containing 125I-MDGF1 on these dishes in the absence of cells. Less than 1% of the MDGF1 was bound to any of the substrates when 20 ng of 125I-MDGF1 were added to 2 ml of medium and the dishes were incubated for 24 h at 37°C.

Laminin, a second basement membrane component, was also increased in relative amount/cell following MDGF1 stimulation of the mammary cells. Using an enzyme-linked immunosassay, laminin was found to increase from 18.5 ± 0.9

FIG. 7. Effects of MDGF1 on collagen turnover. NRK cells were pulse-labeled for 15 min ± MDGF1 with 100 μCi/ml [3H]proline and then either they were harvested for measurements of amino acid incorporation into collagen or the medium was changed to medium free of [3H]proline, and incubation was continued for 24 h. Note the lack of effect of MDGF1 on collagen turnover. There were 4 x 10^6 cells present when pulse labeling was initiated. The data are expressed as counts of collagen/minute/dish.

FIG. 8. MDGF1 effects on collagen mRNA levels in NRK cells. Cells were exposed to 0 or 10 ng of MDGF1/ml for 2 days. Cytosols were prepared, and aliquots were tested for their ability to hybridize to collagen cDNA probes that were nick-translated with 3P-labeled nucleotides. A, cytosol from MDGF1-treated cells; B, cytosol from control cells. Left to right: 10^4, 2.5 x 10^4, 5 x 10^4, and 10^5 cell equivalents of cytosol applied to the nitrocellulose filters. Hybridization probe: α1(R1), 0.05 μg, 20 x 10^4 dpm.

TABLE IV

| Addition          | Cells/dish | Total protein | Collagen |
|-------------------|------------|---------------|----------|
|                   |            | ×10^4         | cpm × 10^4 | cpm       |
| PBS               |            |              |          |
| MDGF1, 12 ng/ml   | 3.1        | 2.0           | 180      |
| MDGF1 concentration | Increase in cell number above control | Differential stimulation of collagen synthesis |
| ng/ml             |            |              |          |
| 0                 | 0          |              |          |
| 2.5               | 23 ± 6     | 2.2 ± 0.1     |          |
| 5.0               | 47 ± 9     | 2.7 ± 0.2     |          |
| 10.0              | 49 ± 5     | 2.9 ± 0.2     |          |

3 W. R. Kidwell, unpublished observations.
Mammary-derived Growth Factor

A mammmary-derived growth factor, MDGF1, has been purified from human milk and an apparently identical protein isotated from human mammary tumors. MDGF1 appears to be distinct from other growth factors in milk. It differs from EGF, a known component of milk (26) as follows. MDGF1 is stable to β-mercaptoethanol reduction, whereas EGF is not (6). MDGF1 is about 10 times as large as EGF and differs by about 0.3 pH units in its isoelectric point (6). It differs from the major fibroblast growth-promoting factor of human milk, HMGFIII, described by Shing and Klagsbrun (7) in molecular weight (62,000 versus ~5,000-6,000). MDGF1 also differs from HMGFI and HMGFII which this author has partially purified from milk (7). The former is about one-half the size of MDGF1, whereas the latter is only about one-fourth as large as MDGF1. Both HMGFI and HMGFII are inactivated by dithiothreitol treatment, unlike MDGF1. MDGF1 is also distinguishable from a bone marrow colony-stimulating factor found in milk. This factor has a molecular weight of 250,000 and a pI of 4.4-4.9 and is reducing agent-insensitive (8).

Preliminary studies with MDGF1 have thus far failed to demonstrate any effects of MDGF1 on bone marrow cell growth in vitro.

A major biological response of mammmary epithelium to MDGF1 is a differential increase in collagen biosynthesis relative to total protein synthesis. Mammmary cells produce only type IV collagen, the collagen type found in the basement membrane on which these cells rest in vivo (27). There is considerable evidence the collagen production is tightly coupled to a mitogenic response of these cells (10), and there are also strong indications that collagen IV biosynthesis is a requisite for mammary cell growth and survival both in vivo and in vitro (1-4).

Responsiveness of the mammary epithelium to MDGF1 is conditional. The cells respond if plated on a plastic or a type I collagen substratum but not on a type IV collagen substratum. We presume this indicates that cell shape differences are manifest on the various substrata and these affect signal transduction when MDGF1 interacts with its receptors on the cell surface. A counterpart to this mechanism may exist in vivo as the mammmary epithelium invades through the basement membrane in response to a proliferative stimulus (2). In this new locus, the epithelium would be dissociated from the basement membrane and consequently be activated to respond to MDGF1 by elaborating a new basement membrane between itself and the stroma. Such a process would be rate-limiting. It is also consistent with the process of generating a basement membrane in vivo since this structure is formed largely at a stromal-epithelial interface and since nonproliferating epithelium which rests on a basement membrane appears to be synthesizing much less type IV collagen than proliferating epithelium (2). Cell shape changes have previously been shown to affect collagen production by smooth muscle cells in culture (28).

Four observations suggest that MDGF1 may be physiologically relevant for the mammary gland. First, human mammary tumors contain a factor which is probably identical to the MDGF1 from milk. This fact suggests that MDGF1 is made by the epithelium. Second, this factor has been shown to stimulate the development of the glandular epithelin

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**Fig. 9. Substratum-dependent responsiveness of mammary cells.** Ducts and alveoli were isolated and cultivated in serum-free medium ± MDGF1 (50 ng/ml) for 3 days. The amount of [%14C]proline incorporated into collagen and non-collagen protein was determined, and the relative responsiveness was calculated as described for Fig. 2. I tissue culture plastic substratum; II, type I collagen substratum; III, type IV collagen substratum.

**Fig. 10. Estrogen effects on MDGF1 responsiveness of mammary cells.** Mammary epithelium was isolated from virgin female mice that had been ovariectomized. One group of animals was given an estrogen implant (20-mg pellet composed of estradiol-17β and cholesterol, 2:1) immediately after ovariectomy. Nine days after ovariectomy, the epithelium from both groups was isolated and cultivated in serum-free medium ± HMGFI and HMGFII which this author has partially purified from milk (7). The former is about one-half the size of MDGF1, whereas the latter is only about one-fourth as large as MDGF1. Both HMGFI and HMGFII are inactivated by dithiothreitol treatment, unlike MDGF1. MDGF1 is also distinguishable from a bone marrow colony-stimulating factor found in milk. This factor has a molecular weight of 250,000 and a pI of 4.4-4.9 and is reducing agent-insensitive (8).

Preliminary studies with MDGF1 have thus far failed to demonstrate any effects of MDGF1 on bone marrow cell growth in vitro.

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Four observations suggest that MDGF1 may be physiologically relevant for the mammary gland. First, human mammary tumors contain a factor which is probably identical to the MDGF1 from milk. This fact suggests that MDGF1 is made by the epithelium. Second, this factor has been shown to stimulate the development of the glandular epithelium in...
whole mammary glands in culture. Third, MDGF1 appears to act synergistically with estrogens since the growth factor does not stimulate mammary cells depleted of estrogen in vivo by ovariectomy. Fourth, a MDGF1-like activity has been detected in a variety of rodent mammary tumors, and its abundance is in rough proportion to the amount of basement membrane present in the tumors (5). However, since NRK cells, a fibroblastic cell line, also respond to MDGF1, it is clear that this factor is not selective for the mammary epithelium. Further experimentation will be required to determine the true target issue for MDGF1 in vivo.

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