Isolation and characterization of epidermal growth factor from human milk

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Received 11 May 1985

Epidermal growth factor (EGF) has been purified from human milk. The purification was monitored with a human placental membrane radioreceptor assay using murine salivary epidermal growth factor I (mEGF I) as a competitive ligand and was achieved exclusively by the use of reverse-phase liquid chromatography (RPLC). The sequential use of preparative, semipreparative and analytical RPLC on an octylsilica support with solvent systems of different solute selectivity such as pyridine formate, triethylammonnium phosphate or perfluorocarbonic acids in the presence of *n*-propanol or acetonitrile allowed purification to homogeneity with 5 consecutive runs. The molecular mass, amino acid composition and NH_2-terminal sequence of human EGF were determined. Gas-phase microsequencing of residues 1–17 revealed the following sequence: Asn-Ser-Asp-Ser-Glu-X-Pro-Leu-Ser-His-Asp-Gly-Tyr-X-Leu-X-Asp which is identical with the NH_2-terminof urogastrone from human urine. The purified polypeptide competes with mEGF for the placental membrane receptor with a k_i of 1 ng. Furthermore, it stimulates the anchorage-dependent as well as -independent proliferation of human and rat indicator cells with half-maximal stimulation at 1 and 2.5 ng/ml, respectively. Although human epidermal growth factor has been unequivocally identified in human milk and – for the first time – shown to be identical with urogastrone from human urine, the high-resolution techniques employed have also revealed the presence of EGF-related molecules which await further characterization. It is possible that EGF and the EGF-related growth factors possess important regulatory functions in normal growth of the human breast during pregnancy and lactation as well as in abnormal growth during mammary tumor formation and progression.

Epidermal growth factor *Human milk* *Microisolation* *Transforming growth factor* *Urogastrone*

1. INTRODUCTION

While working on the purification of nerve growth factor (NGF) from the submaxillary gland of the mouse, Cohen [1] observed that daily injections of certain gland extract fractions into newborn mice produced developmental effects which he could not ascribe to the presence of NGF. These effects included precocious opening of the eyelids and early eruption of the incisors. Using these gross anatomical effects as a biological assay he was able to isolate the factor responsible, a polypeptide which he named epidermal growth factor (EGF) [2] and – a decade later – deciphered its complete amino acid sequence [3].

In 1975, Gregory [4] reported the isolation and characterization of urogastrone, an inhibitory polypeptide of gastric acid secretion present in human urine. Surprisingly, when the primary structure of urogastrone was compared with those of other fully characterized polypeptides a

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

remarkable structural similarity with murine EGF became evident. Of the 53 amino acid residues comprising urogastrone and mEGF, 37 are identical. This homology prompted Gregory to test human urogastrone for eyelid opening activity in mice and mEGF for antisecretory activity in rats and dogs. Since murine EGF inhibited gastric acid secretion and, in turn, urogastrone was capable of inducing eyelid opening in newborn mice, it is generally assumed, although not proven, that both biological activities reside in one molecule, i.e. that in the human urogastrone and EGF are identical [4].

To investigate further the relationship of urogastrone and human EGF, we decided to determine the chemical structure of EGF from a body fluid other than urine as source of urogastrone. Since EGF activity had also been detected in serum, saliva, amniotic fluid and milk [5], the latter fluid was chosen as starting material. Milk is a complex fluid resembling blood in many regards since it contains a large variety of macromolecules including hormones (e.g., adrenocorticotropic hormone, erythropoietin and prolactin) and different cell types such as T- and B-lymphocytes, macrophages, neutrophils and occasionally epithelial cells [6]. The growth promoting activity for human fibroblasts in vitro which is present in milk [7] has been attributed in part to the presence of an EGF-like polypeptide [8] which was later identified in human but not in bovine milk [9].

We report here that acidified milk – upon fractionation on reverse-phase supports – contains 4 polypeptides (A–D) capable of competing with murine EGF for human placental membrane receptors and of inducing DNA synthesis in cultured human skin fibroblasts. In addition, these polypeptides stimulate the replication of rat kidney fibroblasts grown in semisolid media. Hence, they belong to a group of regulatory polypeptides, the transforming growth factors (TGFs), that induce anchorage-independent growth of normal cells the proliferation of which is usually anchorage-dependent [10]. We have purified the polypeptide present in largest quantity (TGF_D) to homogeneity and determined its molecular mass, amino acid composition, partial NH_2-terminal sequence and biological activities. Our results strongly support the assumption that TGF_D is EGF and that human urogastrone and EGF are identical.

2. MATERIALS AND METHODS

2.1. *Human milk*

Samples of human milk were obtained from the Mothers' Milk bank in San Jose, CA and stored at −20°C until use.

2.2. *Cell culture*

Human foreskin fibroblasts were prepared by trypsinization of foreskin and were subsequently passaged in cell culture. Fibroblasts that had been transferred 3 or 4 times after primary cultures were used for DNA-synthesis experiments. Natural rat kidney fibroblasts (NRK, clone 49F) were obtained from the American Type Tissue Culture Association (Rockville, MD). After initial thawing and plating the cells were passaged every 3 days. Fresh cultures were initiated from liquid nitrogen storage every 2 months.

2.3. *DNA synthesis*

Subconfluent monolayers of human foreskin fibroblasts were trypsinized and resuspended in Dulbecco's modified Eagle's medium (DMEM) (glucose concentration 4.5 g/l) containing 5% fetal calf serum (Gibco) at 2.5×10^4 cells/ml. The assay was performed as decribed [11] except that glucocorticoids were omitted and the fetal calf serum concentration raised to 0.4%. The cells were harvested on filter paper using a PHD cell harvester (Cambridge Technology, Cambridge, MA) and DNA synthesis measured by scintillation counting.

2.4. *Colony formation in soft agar*

The soft agar assay was performed in a microculture assay system: aliquots of 250 μl of 0.8% agar (Seaplaque, FMC Labs, Rockland, ME) in DMEM containing 10% fetal calf serum and supplemented with penicillin (100 U/ml), fungizone (0.25 μg/ml), streptomycin (100 U/ml) and glutamine (2 mM) were pipetted into the wells of multiwell tissue culture plates (Falcon, Oxnard, CA). After the agar had solidified, a second 200 μl layer containing 5000 NRK cells in 0.4% agar and DMEM supplemented with 10% fetal calf serum and antibiotics was added. Subsequently, a third 50 μl layer containing the polypeptide to be tested was added. Before seeding, the cell suspension was thoroughly pipetted with a 20 gauge needle to

remove clumps of cells which can cause spurious colony formation. The tissue culture plates were placed in a humidified incubator at 37°C with 5% CO_2 for 10–14 days; rectangular fields of 0.5 cm^2 were then screened for colonies larger than 68 μm in diameter.

2.5. *Polypeptide isolation*

The chromatography system has been described [12]. Reverse-phase columns were either prepared in our laboratory with Lichroprep (RP 8, 2.5 × 6.0 cm, particle size 15–25 μm, pore size 100 Å, Merck, Darmstadt) as reported [13] or obtained prepacked from Brownlee Laboratories (RP 300, 0.7 or 0.46 × 25 cm, particle size 10 μm, pore size 300 Å, Santa Clara, CA). Column eluates were monitored with a fluorometric postcolumn derivatization system [14] and/or by UV absorbance at 280 nm. Mobile phases utilized were 0.36 M pyridine formate (pH 3.0), 0.25 M triethylammonium phosphate (pH 3.0) [15], 0.1% trifluoroaceteic acid (TFA) (pH 2.10) or 0.05% heptafluorobutyric acid pH 2.48) [16]. *n*-Propanol or acetonitrile were used as organic modifiers.

For isolation batches of 400 ml milk were thawed, acidified by the addition of 200 ml glacial acetic acid and centrifuged for 30 min at 100000 × *g*. After centrifugation the pellet containing cellular debris and the creamy top layer were discarded and the resulting liquid filtered through nylon gauze. After adjustment to pH 3.0 with pyridine formate the acidified milk was directly pumped onto the preparative RPLC system.

2.6. *Preparation and iodination of murine EGF I*

Murine EGF I was prepared and iodinated as described [17].

2.7. *Radioreceptor assay for EGF*

Placental membranes were prepared as described by Hirata and Orth [18]. The binding assay was carried out using 400 μl polyethylene tubes; after the addition of 200 μl of a mixture of known standard or unknown sample and ^{125}I-mEGF-I tracer (spec. act. 100 cpm/pg), 100 μl membrane solution was added. After 45 min incubation at 4°C the tubes were centrifuged for 5 min at 7500 × *g* at 4°C and immediately frozen in dry ice/ethanol. The pellet containing the membranes was obtained by cutting the bottom part of the tubes and counted in a Gamma 9000 Counter (Beckman Instruments, Fullerton, CA). In the assay a standard curve was obtained in triplicate in the concentration range of 150 pg to 30 ng per tube. Samples with unknown amounts were determined in duplicates.

2.8. *SDS gel electrophoresis*

Peptides were dissolved in sample buffer and analyzed in a discontinuous slab gel system (20% polyacrylamide) in the presence or absence of 50 mM dithiothreitol [19]. After electrophoresis one half of the gel was cut into 1 mm slices and the polypeptides eluted by shaking overnight at 4°C in 1.0 ml of buffer containing 125 mM Tris-Cl, 1 mM EDTA and 2 mg bovine serum albumin. Two aliquots of 190 μl each were then tested for the presence of active material in the radioreceptor assay. The remainder of the gel was stained with silver [20].

2.9. *Amino acid analysis and sequencing*

Peptide aliquots of 10–20 pmol were subjected to hydrolysis and analyzed with a Liquimat III (Kontron, Zürich) amino acid analyzer [21]. Gas-phase sequence analyses were performed as reported [22] except for the implementation of a new software program obviating the need for a vacuum system in the gas-phase sequencer (designated 02NVAC and supplied by Applied Biosystems, Foster City, CA). Also, the resultant PhNCS-amino acid residues were identified using an improved RPLC separation system suggested by W. Touchstone (Baylor College, Houston) and M. Hunkapiller (Applied Biosystems).

3. RESULTS

3.1. *Purification of TGF_D*

Preparative RPLC of acidified milk (from one individual donor) revealed two radioreceptor active fractions (pools I and II) eluting at 20.4 and 22.2% *n*-propanol (not shown). Both pools were individually rechromatographed in the same solvent system on a semipreparative RP 300 column using a linear gradient from 10 to 18% *n*-propanol. Under these conditions the activity present in pool I was separated into two bioactive fractions eluting at 14.4 and 14.7% *n*-propanol which were designated TGF_A and TGF_B (fig.1, upper).

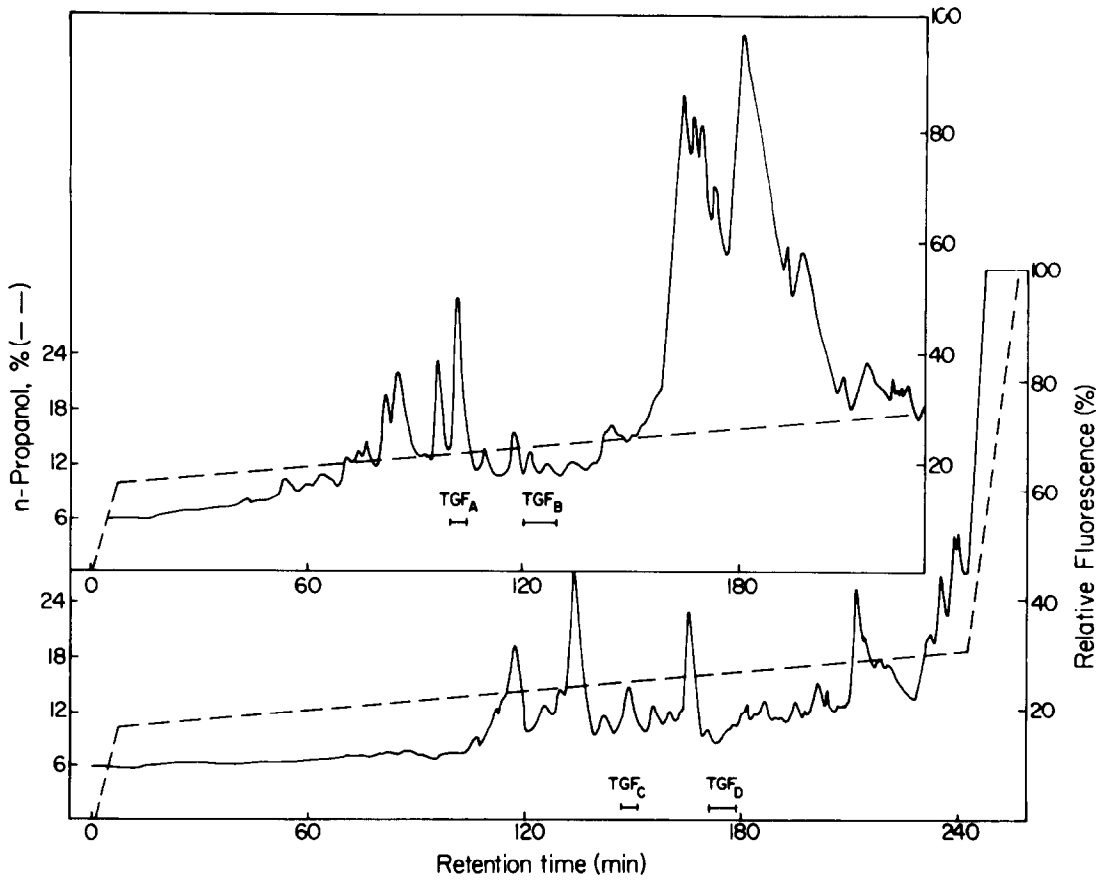

Fig.1. Semipreparative RPLC of pools I and II from preparative column. Pool I (upper trace) and pool II (lower trace) were loaded after 1 : 1 dilution with pyridine formate on a semipreparative RP 300 column in pyridine formate (pH 3.0). The column was eluted with a linear gradient from 10 to 18% *n*-propanol over 240 min. Flow rate was 1.0 ml/min. 3 ml fractions were collected and 30 μl aliquots used for the radioreceptor assay. The horizontal bars indicate the presence of radioreceptor active material.

Chromatography of pool II led to the appearance of two TGFs which eluted at 15.9% (TGF_C) and 16.5% (TGF_D) *n*-propanol (fig.1, lower). The latter molecule was further purified on an analytical RP 300 column using an acetonitrile gradient from 28 to 32% (over 90 min) in 0.1% TFA as mobile phase (fig.2A). The bioactive fractions were pooled and rechromatographed on the same column using triethylammonium phosphate as buffer and eluting the polypeptides with an acetonitrile gradient from 21 to 27% over 120 min. In such a system TGF_D is less hydrophobic and elutes at about 24% acetonitrile (not shown). Final purification was achieved in 0.05% heptafluorobutyric acid where TGF_D is more hydrophobic than in TFA and elutes at about 32% acetonitrile (fig.2B). The molecule was considered better than 90% pure as judged by the appearance of one symmetrical peak in two solvent systems with different solute selectively, i.e. in 0.05% heptafluorobutyric acid and rechromatography in 0.1% TFA.

In addition, the purity was subsequently confirmed by analytical gel electrophoresis and gas-phase sequencing (see below). From 30 g protein present in 3000 ml acidified milk, 12 μg (2 nmol) TGF_D was obtained in highly purified form.

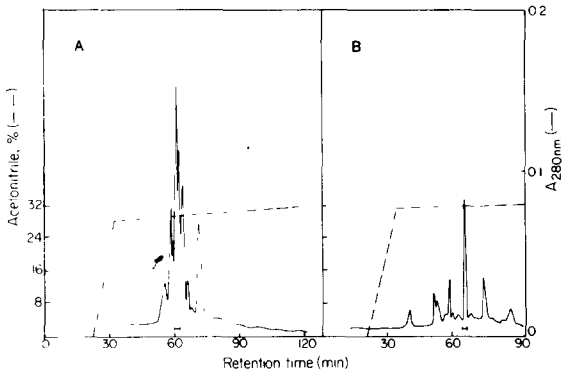

Fig.2. Further purification of TGF_D on an analytical RP 300 column in 0.1% trifluoroacetic acid (pH 2.10) (A) and final purification in 0.05% heptafluorobutyric acid (pH 2.48) (B). Flow rate 0.5 ml/min. 1.5 ml fractions were collected and 15 μl aliquots removed for the radioreceptor assay. The horizontal bars indicate the presence of radioreceptor active material.

Table 1

Amino acid analysis of TGF_D

	TGF_D	Urogastrone[a]
Asx	6.30 ± 0.32	7
Thr	0	0
Ser	3.18 ± 0.75	3
Glx	5.85 ± 0.32	5
Gly	4.33 ± 0.43	4
Ala	2.17 ± 0.87	2
Val	3.56 ± 0.37	3
Met	1.01 ± 0.10	1
Ile	1.91 ± 0.25	2
Leu	5.76 ± 0.91	5
Tyr	5.81 ± 0.89	5
Phe	0	0
His	1.14 ± 0.37	2
Trp	0.92 ± 0.15	2
Lys	2.14 ± 0.42	2
Arg	2.60 ± 0.24	3
Cys[b]	5.36 ± 0.63	6
Pro	2.31 ± 0.23	1

[a] [4]

[b] Determined as cysteic acid

Data are expressed as residues per molecule assuming an M_r of about 6000. Values are means ± SD of 3 determinations and are not corrected for hydrolysis losses

3.2. *Molecular mass, amino acid composition and NH_2-terminal sequence of TGF_D*

Highly purified TGF_D migrated as a single band with an apparent M_r = 6000 in SDS-polyacrylamide gel electrophoresis as determined by silver staining of the gel. The same migration pattern was obtained in the absence of reducing agent. The specific association of this band with radioreceptor competing activity was proven by slicing a parallel unstained gel track that contained unreduced sample. 1 mm slices prepared from this track were extracted overnight and extracts were assayed for EGF competing activity in the receptor assay. All the extracted radioreceptor activity was found to migrate in the 6.0 kDa region of the gel (not shown).

The amino acid composition of TGF_D isolated from human milk is shown in table 1. Values were calculated assuming an M_r of 6000.

Automated Edman degradation of TGF_D was performed with 300 pmol sample (based upon amino acid composition). An initial yield of 79.7% and an average repetitive yield of 82.3% were ob-

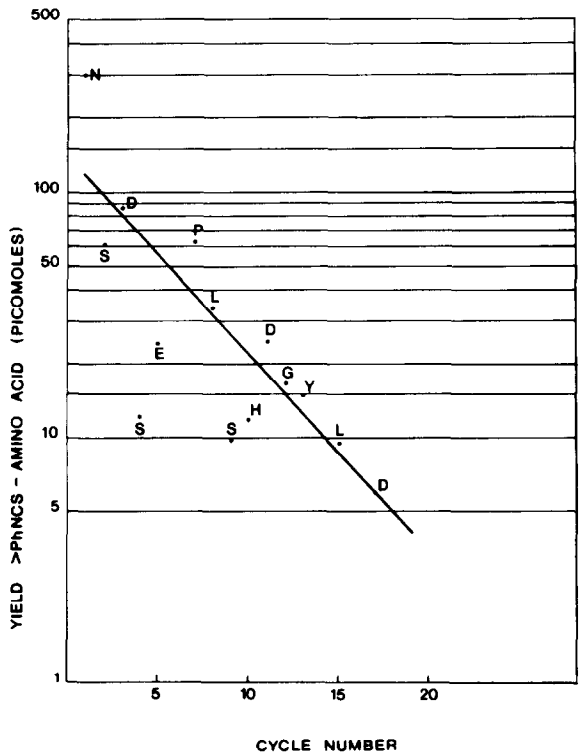

Fig.3. Gas-phase sequence analysis of 300 pmol TGF_D.

tained. Unambiguous identification of phenylhydantoin derivatives of amino acids was possible up to residue 17 (fig.3). Cysteine residues were not positively identified (e.g., by preparing the *S*-alkylated derivatives) in this analysis, but no other residues were identified at cycles 6, 14 and 16. Based on this analysis the partial sequence determined of TGF_D corresponds exactly to residues 1–17 of the sequence of human urogastrone.

3.3. *Biological activities of TGF_D*

RPLC purified TGF_D whose concentration had been determined by amino acid analysis stimulated DNA synthesis in a dose-dependent manner: half-maximal stimulation occurred at a concentration of 1 ng/ml with a 150% increase over control (i.e. with no polypeptide added) at maximal stimulation (fig.4). Because colony formation is a more critical evaluation of growth promoting activity than a system which measures the ability to stimulate [^{3}H]thymidine uptake, the activity of the polypeptide was also tested in an anchorage independent system with rat kidney fibroblasts: half-maximal stimulation was obtained with a concentration of 2.5 ng/ml with the formation of 25 + 5 colonies per 0.5 cm^2 field at maximal stimulation (fig.4). In the placental membrane receptor binding assay, TGF_D caused a dose-dependent displacement of ^{125}I-mEGF-I with a k_i of 1 ng when a concentration range of 0.15–7.5 ng/tube was tested.

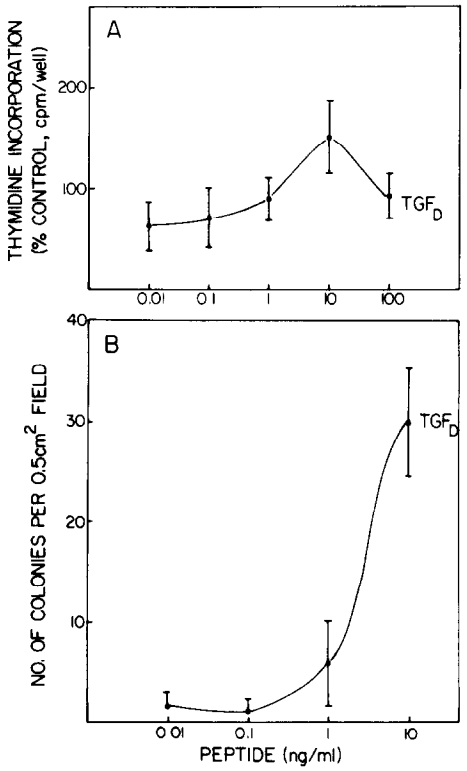

Fig.4. Biological activities of RPLC-purified TGF_D. Effect upon thymidine incorporation in human foreskin fibroblasts (A) and colony formation of rat kidney fibroblasts grown in semisolid media (B). Values are means ± SD of 6 determinations.

4. DISCUSSION

The experiments reported here indicate that human milk contains several polypeptides that are able to compete with mEGF-I for human placental membrane receptors. One of them, TGF_D, has been purified to homogeneity with an approach which is novel in that it is entirely based upon reverse-phase liquid chromatography. The use of solvent systems with different solute selectivity has allowed the rapid isolation of this low-abundance polypeptide from a complex mixture of biological compounds. The amino acid composition of TGF_D is nearly identical with that of human urogastrone. In fact, the small differences in the amino acid compositions of the two polypeptides may be ascribed to inaccuracies associated with the hydrolysis of very small amounts of polypeptide, i.e. less than 20 pmol [21]. Since NH_2-terminal microsequencing of TGF_D revealed a structure identical to that of human urogastrone it is very likely that TGF_D is human EGF and identical with human urogastrone. Our studies also show that human milk contains several other EGF-like transforming growth factors. Similarly, Kimball et al. [23] have recently reported that urine from normal individuals and cancer patients contain at least 5 EGF-related TGFs and that there are distinct differences with regard to the relative levels of these molecules in cancer patients and normal controls. Complete purification and characterization of the TGFs present in human milk as well as urine are necessary to establish the molecular nature of these functionally related factors. Preliminary experiments from our laboratories have indicated that the other TGFs in human milk are biologically and chemically distinct from TGF_D [24].

The origin of EGF and the other EGF-related polypeptides in milk is not yet known. They could be released from the cells present in milk such as macrophages, lymphocytes, neutrophils or epithelial cells. Alternatively, the human breast, a society of interacting epithelial, myoepithelial, stromal connective tissue and vascular bed cells which proliferate when the mammary gland grows to its functional state during pregnancy and lactation could produce and utilize these factors for auto- and paracrine regulation of the proliferative activity of its cells. This idea is supported by the finding that in early lactation a wave of epithelial proliferation is observed [25] and simultaneously colostrum is produced which contains larger amounts of growth promoting activity than milk [8]. As neoplastic transformation is considered by some authors [26] to be a caricature of the renewal or growth of normal tissue it could also be that the transforming growth factors in milk (including EGF) play an important role in mammary tumor development, for instance through their inappropriate or constitutive expression by the neoplastic cell(s). In agreement with this possibility is the recent identification of EGF-like transforming growth factors and of EGF-receptors in human breast cancer cells [27].

Efficient microtechniques such as the one presented here should facilitate the characterization of such growth regulating factors from breast milk, mammary tumor tissue as well as tumor cell lines and allow one to define in molecular terms their relationship as a basis for a better understanding of the (normal) proliferation of human breast cells during pregnancy and lactation and the (abnormal) proliferation during breast tumor formation and progression.

ACKNOWLEDGEMENTS

We thank Alan Maxfield, Roland Schroeder and Thomas O'Toole for their excellent technical assistance. We are also grateful to Mrs Teresa Asquith (San Jose Mothers Milk Bank) and Dr Philip Sunshine (Stanford Medical School) for the human milk samples. This work was supported by grants from the National Institutes of Health (AM 18811, HD 09690, AM 25434, NS 04270) and Cooper Biomedical, Palo Alto, CA.

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