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Modulation of Extracellular-Matrix Synthesized by Cultured Stromal Cells From Normal Human Breast Tissue by Epidermal Growth Factor

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A routine, reproducible procedure was developed for the preparation and characterization of stromal cells from normal human breast tissue obtained by reduction mammaplasty. Isolates (n = 15) all exhibited enhanced rates of proliferation, even in the presence of 20% fetal calf serum, when exposed to epidermal growth factor or transforming growth factor$_\alpha$ (both 10^{-8} M). Cellular responsiveness to these growth factors was consistent with expression of specific surface receptors for epidermal growth factor ($\sim 10^4$/cell). In cultures, stromal cells elaborated an extensive, cross-linked, insoluble extracellular matrix which remained firmly associated with the plastic surface of tissue culture ware upon lysis of cells. The insoluble matrix material was analyzed using enzymatic digestion procedures following incorporation of radiolabelled precursors into macromolecular material prior to lysis and preparation. The relative proportion of glycoconjugate (glycopeptides and proteoglycans) and collagenous material present in matrix material was ~45% and ~55%, respectively, and this was modulated by inclusion of epidermal growth factor into culture medium to ~60% and ~40%, respectively. Under similar culture conditions stromal cells synthesized twice as much hyaluronate as was produced by control cultures. By use of specific antibody preparations we identified at least four species of glycopeptide present in stromal matrices (namely, fibronectin, laminin, tenascin, and thrombospondin) as well as three types of collagen (types I, III, and IV). The rapid and reproducible procedure for the preparation of radiolabelled insoluble matrix material from normal human breast tissue allows for the study of cellular interaction involving extracellular matrix turnover and degradation.

Key words: collagens, glycoconjugates, TGF$_\alpha$, fibronectin, tenascin, laminin thrombosopondin

The extracellular environment plays a key role in growth and differentiation of many cell types, and perturbation of this environment leads to modulated phenotypic expression [1–4]. It has been reported that growth factors are capable of significantly changing the elaboration of the extracellular matrix and may therefore so influence

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matrix mediated processes [3,5,6]. In breast tissue, which in non-lactating females consists predominantly of connective tissue and fat, the stromal cells make up a population of "fibroblast-like" cells that reside on the side of the basal lamina opposite that occupied by both the luminal and myoepithelial cells [7–9]. The latter are considered to be responsible for the biosynthesis and elaboration of the basement membrane whose main constituents are type IV collagen, laminin, and heparin/heparan sulphate [10,11]. This combination of cells (stromal and epithelial) has been shown to produce the flexible substrate which appears essential for the latter's full differentiation, at least in the rodent systems [12–14]. Stromal cells are, however, responsible for the biosynthesis and secretion of the bulk of the connective tissue matrix surrounding the ducts and alveoli of the breast [7]. Haslam [15] has suggested that stromal cells potentiate the effects of hormones, for example, 17-β-estradiol, on cultured mammary epithelial cells. In addition it has been shown that even minor modifications in the synthesis of mesenchymal connective tissue molecules can be correlated with marked changes in the morphology and differentiative pattern of epithelial cells in vivo [16–18]. Furthermore, Bernfield and Bannerjee demonstrated that extracellular matrix turnover by epithelial and mesenchymal cells was essential for growth and morphogenesis [19] and that basement membrane glycosaminoglycan catabolism preceded these events.

Cell suspensions prepared by enzymatic disaggregation of breast tissue consist of a number of different cell types, including luminal and myoepithelial cells, stromal cells, adipocytes and vascular smooth muscle, and endothelial cells [20,21]. We have isolated and characterised breast stromal cells (hBST) from human tissue. We report our findings on the biosynthesis and secretion of extracellular matrix (ECM) macromolecules by hBST cells. Furthermore, we have studied the effects of epidermal growth factor (EGF) and transforming growth factor$_\alpha$ (TGF$_\alpha$) on the elaboration of ECMs by such cultures. Our in vitro model allowed us to assess the effects of EGF and TGF$_\alpha$ on the growth and matrix elaboration of stromal cells isolated free from the epithelium which may influence their responses.

MATERIALS AND METHODS

Materials

Most chemicals and media for tissue culture including fetal calf serum were obtained from Amimed AG (Basel, Switzerland). EGF and TGF$_\alpha$ were supplied by Collaborative Research Inc. (Bedford, MA). DNase I (EC 3.1.21.1), collagenase (EC 3.4.24.3), trypsin (EC 3.4.21.4) and testicular hyaluronate 4-glycanohydrolase (EC 3.2.1.35) were supplied by Sigma Chemical Co. (St. Louis, MO). Radioisotopes, including [methyl-^{3}H]-thymidine (92Ci/mmol), D-[6-^{3}H]-glucosamine (22Ci/mmol), [2-^{3}H]-glycine (19 Ci/mmol), L-[6-^{3}H]-fucose and [^{35}S]-labelled immunoglobulins were supplied by Amersham (Buckinghamshire, England). Enzymes used for glycosaminoglycan (GAG) analysis were from Miles (Naperville, IL) and included chondroitin ABC lyase (EC 4.2.2.4) and hyaluronidase from *Streptomyces hyaluronolyticus*. All antibodies and reagents used for immunocytochemical characterisation of cultured cells were obtained from Dakopatts, IG-Instrumenten Gesellschaft (Zurich, Switzerland) except for 3,3-diaminobenzidine HCl, which was from Serva (Heidelberg FRG). Polyclonal antibodies (rabbit) against mouse tenascin and type IV collagen were generously provided by Dr. Chiquet-Ehrismann at the Friedrich Miescher Institute [22]. Polyclonal antibodies (rabbit) against human collagen, types I and III, and laminin were

purchased from Heyl Chemisch-pharmazeutische Fabrik (Berlin, FRG). Monoclonal antibodies against human thrombospondin were supplied by Seratec, (Cambridge, UK). Rabbit polyclonal antibodies to human tissue (non-serum) fibronectin were obtained from Calbiochem, LaJolla, CA). Other chemicals and reagents were from E. Merck (Darmstadt, FRG).

Methods

Cell culture. Normal human breast tissue was obtained from reduction mammaplasty and trimmed of all visible fat. Tissue was cut into 1 cm^2 cubes and washed thrice with phosphate buffered saline (PBS) containing penicillin, streptomycin, and fungizone (200 U/ml, 200 U/ml, and 5 μg/ml, respectively). After the third wash, tissue was finely minced before digestion for 8 h in enriched media (IMEM-ZO Gibco, Basel, Switzerland, with 2 mg phenol red/litre) consisting of 20% fetal calf serum (FCS), penicillin and streptomycin both at 100 U/ml, nonessential amino acids (NEAA), MEM vitamins, 15 mM HEPES (pH 7.3), 7 ng/ml Naselenite, and 0.5 μg/ml hydrocortisone, containing 0.1% collagenase (w/v) and 0.1% hyaluronidase (w/v). At the end of the incubation period digests were exposed briefly to DNase I (100 U/ml) to reduce viscosity resulting from DNA released from broken cells. We found that the latter step facilitated the pelleting of small cell aggregates and single cells by centrifugation (600g/10 min). Cell pellets were resuspended in IMEM-ZO and plated into culture flasks. Isolates were maintained for approximately 14 days with regular medium changes and then selectively trypsinized as described [23]. After passaging cells were weaned onto complete IMEM-ZO media containing less FCS (15%) and without NEAA and vitamins. They were subsequently passaged at a split ratio 1:5 every 14 days by trypsinization (0.05% w/v, 2 mM EDTA) and used for experiments through fourth passage. Stocks were frozen in liquid nitrogen at first passage.

Immunohistochemical characterization of cultures. Isolates of stromal cells were fixed in methanol prechilled to −20°C and then characterized using specific antibodies to α-actin, factor VIII, vimentin, desmin, and cytokeratins to assess levels of possible contamination by smooth muscle cells, endothelial cells, and/or epithelial cells with help of, and according to the methods described by, Petersen and van Deurs [20,21].

Growth kinetics studies. Cells were plated in complete media in 12 well plates (Costar) at a density of 4.5×10^4 cells/cm^2. Twenty-four hours after seeding one plate was trypsinized and cell numbers determined using a Coulter counter. In the remainder, medium was replaced with complete medium or complete medium containing either 10^{-8} M EGF or 10^{-8} M TGF_α. Cell numbers were determined on plates from control and treated cultures every 24 h.

Nuclear labelling. Cells were plated into 35 mm petri dishes at a density of 1.25×10^4 cells/cm^2 in complete medium. After 48 h this medium was replaced with IMEM-ZO/BSA/HEPES containing 0.1% w/v BSA and 15 mM HEPES. Cultures were maintained for 24 h and then treated for a further 24 h with the IMEM-ZO/BSA/HEPES containing [^{3}H]-methyl-thymidine (1 μCi/ml) and either EGF, TGF_α (both at 10^{-8} M), or 20% FCS. Labelled cells were fixed with 25% acetic acid in ethanol for 10 min and dried. Cell layers were then coated with Ilford L_4 liquid photographic emulsion and developed 24 h later. After development plates were stained with Mayer's hemalun solution. The number of radioactively labelled nuclei were counted and expressed as

percentages of total nuclei from a series of randomly selected microscopic fields, such that the total nuclei counted exceeded 1,000/dish.

Binding of [^{125}I]-epidermal growth factor. Confluent cultures maintained on serum-free medium for 48 h were used for saturation binding studies and the procedures used were exactly as described previously [24]. Nonspecific binding was determined by inclusion of excess (500 ng/ml) unlabelled growth factor to parallel wells and was always less than 10% of total cell-associated radioactivity. Binding parameters were obtained for each individual experiment by computerized weighted nonlinear curve-fitting analysis [25].

Extracellular matrix production and induction. Cells were plated into 12 well multiwell plates in complete medium as described above (4.5×10^4 cells/cm^2). After 24 h, medium was replaced with the same containing either 10^{-8} M EGF or TGF_α and 50 μg/ml Na-ascorbate (ASC). Medium was changed every 3 days and fresh ASC was added daily. After the requisite time in culture, cells were rinsed twice with double-distilled water and lysed with 25 mM NH_4OH as described by Scott-Burden et al. [26]. Insoluble cross-linked matrices, which remained associated with the culture dish surface, were rinsed three times with 70% aqueous ethanol and air-dried overnight. To study matrix induction, cultures were maintained in the presence of either [^{3}H]-glucosamine or [^{3}H]-glycine for the 24 h preceding each time point. At the time of matrix harvest cell numbers were also determined on parallel cultures using a solution of 0.05% trypsin and collagenase (125 μg/ml) dissolved in PBS for preparation of single cell suspensions and counted as described above. Air-dried, radiolabelled ECMs were solubilised by overnight incubation with 2 M NaOH at 37°C and aliquots counted using Instagel (Packard) in a Packard 460C scintillation counter, after neutralization with glacial acetic acid [26].

Compositional analysis of extracellular matrix. The preparation of extracellular matrices for enzymatic compositional (HTC) analysis followed the same procedure as outlined above except that cultures were maintained in the presence of either [^{3}H]-glucosamine, [^{3}H]-glycine, or [^{3}H]-fucose for the final 72 h growth period. Isotopes were present at the level of 5 μCi/ml. After harvest and drying, ECMs were digested with hyaluronidase (H), trypsin (T), and collagenase (C) sequentially. Proteolytic enzymes were dissolved in 25 mM Tris HCl (pH 8.0) containing 5 mM $CaCl_2$ and used at a concentration of 20 μg/ml. Hyaluronidase (H'ase) isolated from ovine testes was dissolved in 50 mM Tris acetate (pH 6.0) containing 0.1% BSA and used at 20 μg/ml. After each incubation (37°C for 4 h), samples consisting of one-half the digest volume were collected and counted as described above. This procedure is essentially the same as that previously validated for compositional analysis of ECM from vascular smooth muscle cells vascular endothelial cells and fibroblast cultures [26,27] and has been adapted for the studies reported here (see results and discussion).

Glycosaminoglycan analysis. Extracellular matrices were prepared as above from cells cultured in 100 mm petri dishes in the presence of [^{3}H]-glucosamine and/or [^{35}S]-Na_2SO_4 for the final 72 h before harvest. After preparation of ECMs (as described above) samples were scraped into 1 ml of PBS containing 100 mg/ml papain and incubated at 65°C for 1 h. Digests were dialysed for 24 h with repeated changes against 10 liters of double-distilled water, and insoluble material was removed by centrifugation in an Eppendorf microcentrifuge. The solubilised GAG samples were digested with hyaluronidase (1 mg/ml) from *S. hyaluronolyticus* or chondroitinase ABC in the

appropriate buffer for 3 h at 37°C [26]. Alternately, samples were treated with nitrous acid [28] and incubated at room temperature for 3 h. Paper chromatography [29] was used to separate digested from undigested material and compared with the control samples incubated in the absence of degradative enzymes [29]. The amount of undigested material remaining at the origin in relation to the total amount of material digested by all three digestive processes was used as the basis for determining the percentage of total GAGs sensitive to nitrous acid, hyaluronidase, or chondroitinase digestion [26,29]. Greater than 90% of the material was sensitive to digestion in a combined sequential digestion (hyaluronidase followed by chondroitinase ABC followed by nitrous acid). Hyaluronidase digestions were performed under conditions that favoured digestion of non-sulfated N-acetyl glucosamine containing GAGs [30].

Matrix characterization using specific antibody binding. ECM-coated dishes, which were prepared from cultures maintained in the presence of [^{3}H]-glycine for the final 24 h, were gently shaken with a solution of 0.1% BSA in PBS (PBS-BSA) for 2 h. This solution was replaced with either non-immune sera or specific antibody at an optimal dilution (usually 1:500) also in PBS-BSA and samples were shaken for a further hour. After rinsing three times with a solution of 0.05% Tween-20 in PBS (PBS-Tween), [^{35}S]-labelled second antibody diluted in PBS-BSA was bound to matrix layers and subsequently rinsed with PBS-Tween as above. ECM/antibody complexes were dissolved in 2 M NaOH, neutralized in glacial acetic acid, and counted as described above.

RESULTS

Characterization of Human Breast Stromal Cells

Human breast stromal cells (hBST) were isolated by enzymatic disaggregation of breast tissue obtained from reduction mammaplasty and selective trypsinisation [23] as described in Methods. The hBST cells stained positive for vimentin only (>95%) and showed no staining by immunoperoxidase procedures for smooth muscle α-actin and cytokeratin as compared to positive controls of human, vascular smooth muscle, and breast epithelial cells (Fig. 1). Stromal cell preparations did not exhibit cytochemical staining when exposed to monoclonal antibodies raised against endothelial cell-surface factor VIII/Von Willebrand factor antigen (data not shown). Identification of cells derived from breast tissue as "stromal cells" by such criteria has already been described [18]. Cells grew continuously in culture to form multilayers and showed no attenuation of growth on reaching confluency. Stromal cells were shown to possess specific EGF receptors (1.4×10^4/cell) by radioligand techniques and this was unaffected by passaging of cultures (Fig. 2). Receptor affinities for EGF were determined following Scatchard analysis to be ± 3 nM.

Growth and Mitogenesis Studies

Cells (hBST) exhibited a doubling time of 24–36 h (Fig. 2) in culture and proliferation rates could be correlated with plating density (data not shown). Supplementation of media with EGF or TGF_α (10^{-8} M) markedly enhanced proliferation rates of cultures, even in the presence of FCS (20%) as compared to control cultures (Fig. 3A). Under serum-free conditions both EGF and TGF_α were found to be mitogenic to hBST cells as assessed by [^{3}H]-thymidine nuclear labelling procedures (Fig. 3B). The stimulation in DNA synthesis by both agonists was $\pm 50\%$ of that observed with 20% FCS.

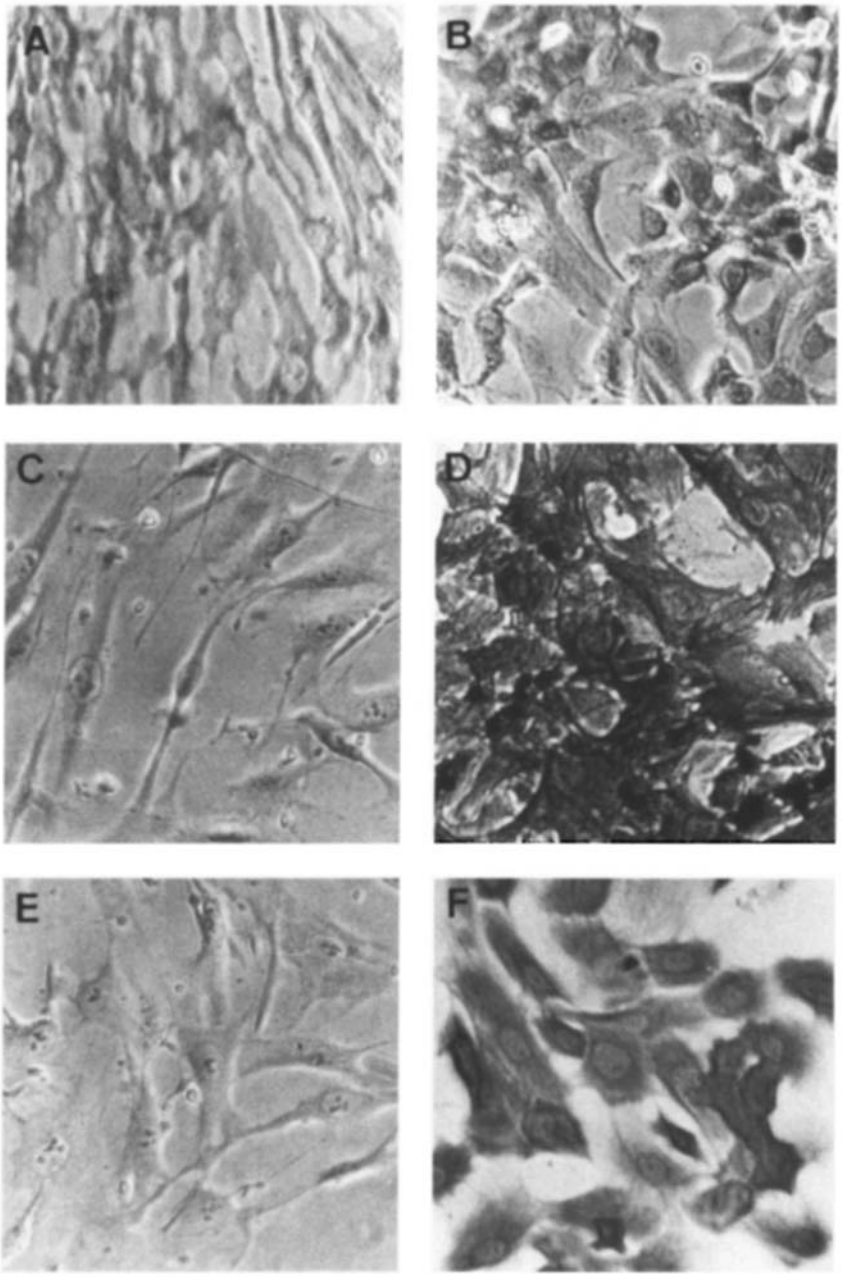

Fig. 1. Phase contrast micrographs of Giemsa stained, methanol fixed hBST (**A,C,E**), vascular smooth muscle (**B,D**), and human breast epithelial cells (**F**) after immunocytochemical procedures (Methods). Cells were incubated with monoclonal antibodies raised against vimentin in A and B; against α-smooth muscle actin in C and D; and against epithelial specific cytokeratins in E and F. $\sim \times 300$.

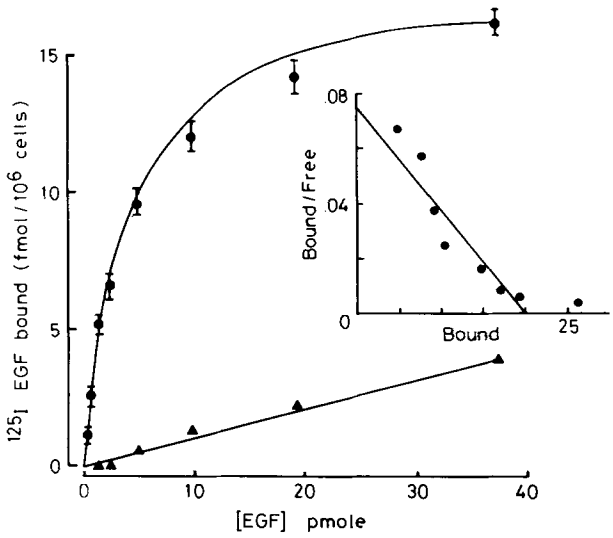

Fig. 2. Binding of [^{125}I]-epidermal growth factor binding to hBST cells. Specific binding of the radiolabelled ligand was performed at 4°C as described under Methods, and the data points for specific binding (●—●) shown in the saturation profile are the mean values (±SD) from three separate experiments. Determination of non-specific binding (▲—▲) was performed as described (Methods). The Scatchard plot insert was obtained by transformation of saturation binding data from one of these experiments by computer analysis as described in Methods.

Kinetics of Extracellular Matrix Production

The onset of matrix production by cultured hBST cells occurred when cells reached confluency, at a density of approximately 8×10^4 cells/cm^2 and continued in a linear manner with time in culture (Fig. 4). The incorporation levels of [^{3}H]-glycine into ECMs was significantly ($P > 0.001$) reduced by the inclusion of EGF in the culture medium (Fig. 4A). However, the converse was true for the incorporation of [^{3}H]-glucosamine into matrix material after 11 days in culture, which suggested that prolonged exposure hBST cells to EGF might stimulate their biosynthesis of matrix glycoconjugates. Some cultures were maintained for up to 15 days in the presence of EGF, and under such conditions the amount of [^{3}H]-glucosamine incorporated into matrix material was twice that of control (unsupplemented) cultures (data not shown). We obtained essentially the same results in similar experiments (n = 2) conducted with TGF_α (data also not shown).

Compositional Analysis of Extracellular Matrices by Sequential Enzymatic Digestion

Extracellular matrix material elaborated by hBST cells was analysed by sequential enzymatic digestion as described under Methods. This type of procedure, which has been described previously, relies on the specificity of the enzymes employed [26,27]. In preliminary experiments we established that hBST did not synthesize matrix material sensitive to elastase digestion [presumably elastin, 26,27,31,32] and we wished to gain more insight regarding glycoconjugate (trypsin sensitive) components of the ECM elaborated by our cells. We therefore modified the published procedure [27] by the use of

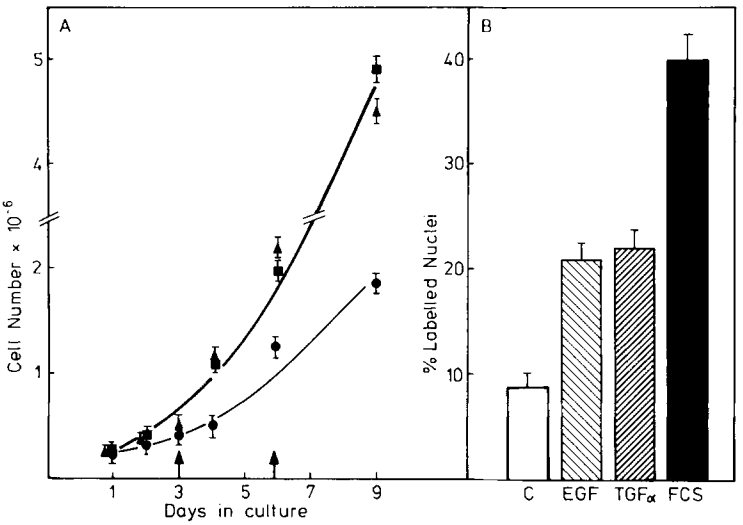

Fig. 3. Growth kinetics of hBST cells in culture. The assay of the growth kinetics of hBST cells (**A**) and the determination of their mitogenic activity, by nuclear labelling, on exposure to EGF or TFG_α (**B**) was performed as described (Methods). For growth experiments, cells in normal media were seeded at 1.25×10^4 cells/cm^2 in multiwell plates. After 24 h cell numbers were determined and media changed to fresh media with (▲—▲, ■—■) or without (control, ●—●) the addition of EGF (▲) or TGF_α (both 10^{-8} M). Routine medium changes (↑) were performed thereafter and cell numbers determined daily. The data represent the means and ± standard deviations (SD) of triplicate cell number determinations, and experiments were performed thrice. Mitogenesis assays were performed exactly as described under Methods, and the data are represented as the % of total nuclei labelled by [^{3}H]-thymidine. Determinations were performed at least three times and values are the means ± SD of the values obtained; <1,000 nuclei were counted for determinations.

hyaluronidase (H'ase) as a first step in our method, and some data pertaining to the validation of our modified procedure are shown in Figure 5. Digestion of matrix material elaborated by hBST cells in the presence of [^{3}H]-glycine by H'ase released only 5–8% of the total incorporated radioactivity even after prolonged incubations with the enzyme (Fig. 5A). In contrast digestion with trypsin (Fig. 5B) followed by collagenase (Fig. 5C) resulted in the solubilisation of ±45% and ±50% of the total matrix associated radioactivity, respectively. These results are consistent with the enzyme specificities and the polypeptide nature of the radiolabelled substrate components in this instance. Digestion of matrices, elaborated by hBST in presence of [^{3}H]-glucosamine, by H'ase released ±30% of total radioactivity in 4 h of incubation (Fig. 5A). Most of the remaining radioactivity was solubilised by incubation with trypsin (Fig. 5B). When [^{3}H]-fucose was used as the radioactive precursor only trypsin digestion of matrices released appreciable amounts (±90%) of the label in keeping with the fact that this carbohydrate, unlike glucosamine, is mainly associated with matrix glycoproteins and not proteoglycans/glycosaminoglycans and also does not appear to be a sugar found in the carbohydrate moeities of the interstitial collagens [33].

In order to investigate the effects of medium supplementation by EGF or TGF_α on matrix elaboration by hBST, we performed enzymatic compositional analysis as described in Methods and discussed above. The relative proportion of polypeptide-material synthesised by control (unsupplemented) cultures in the presence of [^{3}H]-glycine, as precursor, which was glycoconjugate (H'ase + trypsin) and collagenous material was

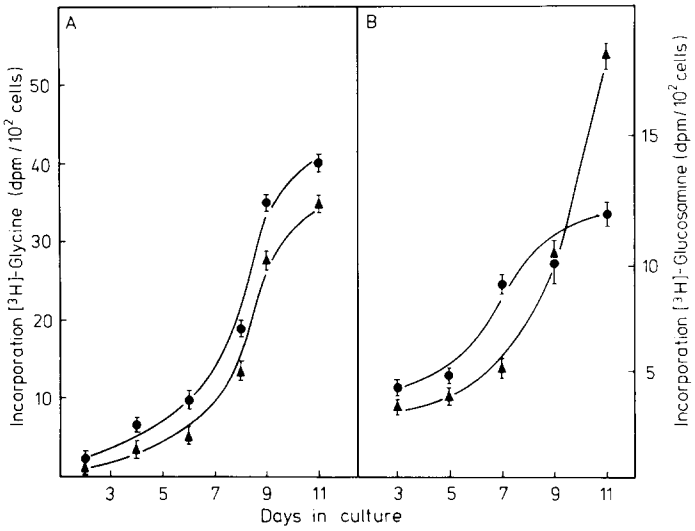

Fig. 4. The kinetics of matrix elaboration by hBST cells during culture. Cells were seeded and exposed to EGF (▲—▲), where appropriate, as described under Figure 3. Cultures were supplemented daily with ascorbic acid and with [^{3}H]-glycine (**A**) or [^{3}H]-glucosamine (**B**) for 24 h prior to matrix isolation. Parallel cultures were used to determine cell numbers at the requisite times of experiments. Radiolabelled, dried matrices were solubilised and counted by liquid scintillation counting as described (Methods). Determinations were performed on triplicate samples, and experiments were repeated thrice; the values shown represent the means ± SD.

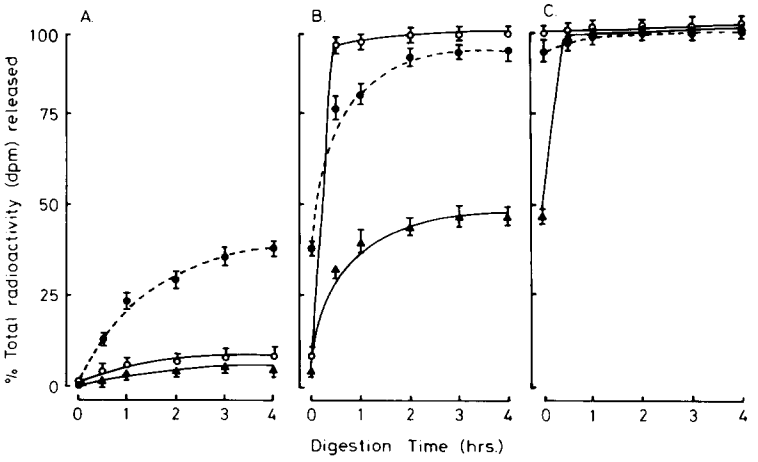

Fig. 5. The kinetics of sequential matrix digestion by specific enzymes. Validation of the enzymatic analysis procedure (HTC). Cells (hBST) were grown in the presence of ascorbic acid for 11 days and labelled for 24 h prior to matrix isolation with [^{3}H]-glycine (▲—▲), [^{3}H]-glucosamine (●---●), or [^{3}H]-fucose (○—○). Radiolabelled ECMs were digested sequentially with H'ase (**A**), trypsin (**B**), and collagenase (**C**) in 3 sequential 4 h periods of incubation at 37°C (Methods). Aliquots were removed and counted at the times indicated (30 min, 1 h, 2 h, 3 h, and 4 h). Determinations were performed at least three times, and values represent means ± SD.

48% and 52%, respectively. When the same type of analysis was performed on ECMs from cultures supplemented with EGF or TGF_{α}, in presence of [^{3}H]-glycine, the relative proportions of glycoconjugate and collagenous material were ±60% and ±40%, respectively (Fig. 6A). Thus, the growth factors effectively reduced the relative amount of collagen present in the matrix as compared to control matrices. We repeated such analytical procedures on matrices elaborated by control and supplemented cultures in the presence of [^{3}H]-glucosamine as precursor and predictably in all cases ±90% of the total radioactivity was released by sequential H'ase and trypsin digestion (Fig. 6B). However, whereas the relative proportions of the radioactivity which was solubilised by H'ase and trypsin were ±30% and ±60%, respectively, for "control matrices," the amount of H'ase-sensitive material in matrices from growth factor supplemented cultures was >65% (Fig. 6B). These data suggested that growth factor supplementation increased synthesis of matrix associated glycosaminoglycan material that was not covalently bound to peptide. Since hyaluronic acid is the only such compound identified in mammalian systems [30,34], we next analysed the glycosaminoglycan composition of the matrices elaborated by hBST cultures in the presence and absence of EGF.

Glycosaminoglycan Analysis of hBST Matrices

The principal GAG chains associated with the matrices of both control and supplemented (EGF) cultures were of the N-acetyl-galactosamine class, namely, chondroitin sulphates (±60% of total matrix GAG, Table I). Cells maintained in presence of EGF for 9 days elaborated matrices containing relatively twice as much hyaluronate (±30% vs. 16%) as their control counterparts (Table I). Since at this stage of growth, control and growth factor supplemented cultures incorporated similar total amounts of

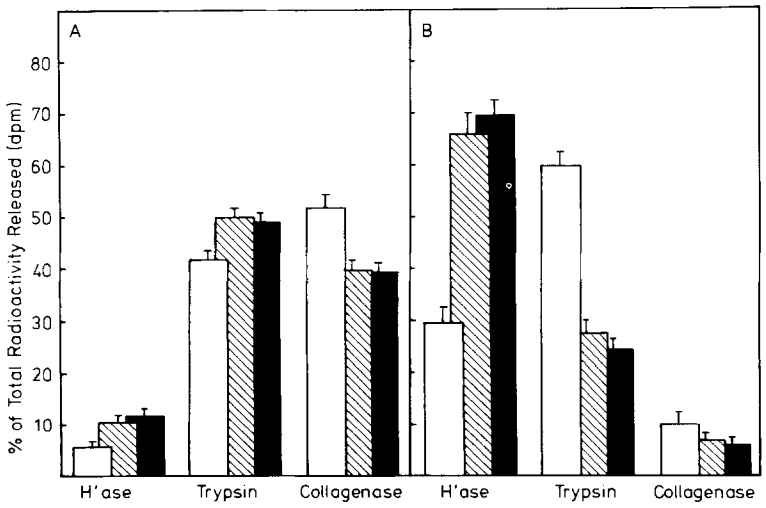

Fig. 6. Sequential enzymatic digestion (HTC) of extracellular matrix elaborated by hBST cells in the presence or absence (□) of either EGF (▨) or TGF_{α} (■). Cells were cultured and matrices prepared as described under Methods and Figure 3. HTC analysis was performed on matrices from cells cultured in the presence of **A)** [^{3}H]-glycine or **B)** [^{3}H]-glucosamine and the data (means ± SD) expressed as the amount (%) of radioactivity released by specific individual enzyme digestion in relation to the total radioactivity released by sequential enzyme digestion.

TABLE 1. Glycosaminoglycan Analysis of Matrices Elaborated by hBST Cells*

Glycosaminoglycan chain species	Composition (%)	
	Control ECM	EGF ECM
Hyaluronic acid	16 ± 0.5	30 ± 0.2
Chondroitin sulfate	63 ± 1.0	58 ± 2.0
Heparin/heparan sulfate	21 ± 3.0	12 ± 1.0

*The analysis of glycosaminoglycan chains associated with matrix proteoglycans synthesised by cultured hBST cells grown in the presence and absence of EGF (10^{-8} M) was performed as described under Methods. Cells were grown in 100 mm culture dishes for 8 days with normal routine medium changes and for the final 48 h of culture cells in presence of [^{8}H]-glucosamine (Methods). The data are represented as the percentage of material that is degraded by specific enzymatic or chemical procedures (Methods) relative to the total degradable material when samples were digested sequentially by H'ase, chondroitinase, and finally HNO_2. Typically, in experiments (n = 3) aliquots taken for degradation prior to paper chromatography contained from 28,000 to 43,000 dpm, and <90% were degraded by the digestive procedures in combination (Methods).

[^{3}H]-glucosamine into matrix material (Fig. 4B), the amount of hyaluronate present in the matrices of supplemented cultures was double that of control cultures. At later times in culture this effect was still further pronounced such that up to 40% of the glycosaminoglycan content of the matrices from EGF supplemented cultures was sensitive to pure hyaluronidase (data not shown). The relative proportions of heparin/heparan sulfate and chondroitin sulfate were also decreased in matrices from EGF supplemented cultures (Table I).

Specific Antibody Binding to hBST Matrices.

In order better to assess the data we obtained using the sequential enzymatic digestion procedure on ECMs labelled with [^{3}H]-glycine (Fig. 6A), we utilised a number of specific antisera to identify and assess the relative proportions of some of the glycopeptides and collagen molecules. Antisera saturation binding profiles were first performed to determine optimal levels for each preparation (data not shown), and then the amount of antibody bound to individual matrix samples was determined by the use of [^{35}S]-labelled second antibodies (Fig. 7). The data, which are expressed as a ratio of the amount of antibody bound ([^{35}S] dpm)/the amount of matrix material ([^{3}H]-glycine dpm), indicated that there was an overall decrease in the amounts of matrix associated fibronectin and tenascin produced by EGF supplemented cultures as compared to controls and, furthermore, that in both cases fibronectin was the predominant glycopeptide synthesised (Fig. 7A). Also in the case of EGF supplemented cells, there was a decrease in the amount of type IV collagen associated with the matrix they elaborated (Fig. 7B); this is consistent with the data obtained by the enzymatic analysis procedure (Fig. 6A), where we observed a relative decrease in the amount of collagenous material synthesised by such cultures.

DISCUSSION

The identification of cells isolated from human breast tissue as "stromal" is complicated by the fact that within such biopsies there are a variety of cellular phenotypes [20,21]. The immunocytochemical procedures used in our studies have been documented previously by Petersen and van Deurs [21] and with their assistance and

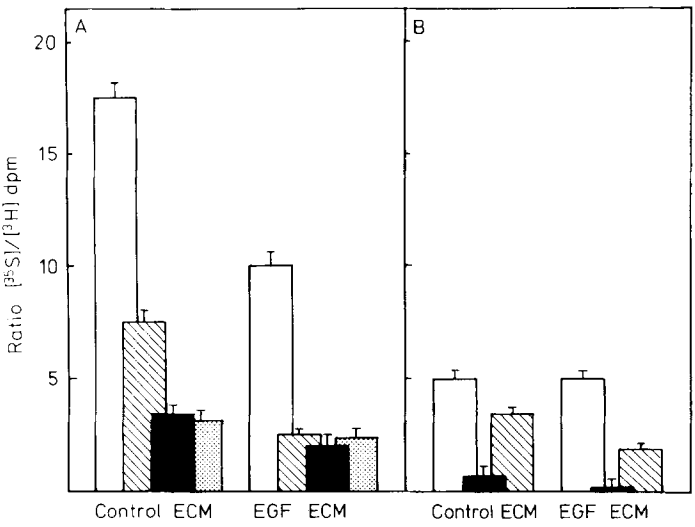

Fig. 7. Specific antibody binding to extracellular matrices elaborated by hBST cells in presence or absence of EGF (10^{-8} M). Extracellular matrices were prepared from hBST cultures maintained in the presence of [^{3}H]-glycine for the final 24 h before isolation as described under Methods. Specific antibody binding was performed as outlined in methods using [^{35}S]-labelled second antibodies. Following binding and washing of matrix material samples were solubilised by 2 M NaOH and radioactivity determined as outlined under Methods. Data are expressed as the ratio of [^{35}S]/[^{3}H]. Determinations were performed in triplicate and values represent means ± SD. Specific antibody binding to matrices was performed as shown in **A**, using α-fibronectin (□), α-tenascin (▨), α-laminin (■), and α-thrombospondin (▤), in **B**, using α-collagen type I (□), α-collagen type III (■), and α-collagen type IV (▨).

methodology we were able to distinguish clearly and isolate the stromal phenotype. The multilayer growth pattern of the stromal cells in culture is reminiscent of that observed for cultured vascular smooth muscle cells [27]; however, primary cultures of hBST cells were immunologically negative for both smooth muscle specific α-actin and desmin. Furthermore, hBST cells exhibited enhanced responsiveness to EGF both in the absence and presence of 20% FCS (Fig. 3) and expressed elevated levels of specific cell surface, high affinity receptors for the ligand (Fig. 2). Vascular smooth muscle cells respond poorly to this growth factor and possess far fewer cell surface receptors for EGF [24]. One final criterion which may be invoked for the identification of phenotypes is the biosynthesis of specific polypeptide(s); in this regard, cultured smooth muscle cells have been shown to synthesise elastin [22,27,31,32]. We could find no evidence for the biosynthesis of the latter by cultured hBST. The enzymatic procedure we used to analyse the polypeptide composition of matrices elaborated by hBST cells in culture, both in the presence and absence of EGF or TGF_α, was tested for its validity as shown (Fig. 5). The original procedure upon which our modified method was based relied on both the specificity of the enzymes employed and the sequence in which they were used [27]. We approached the development of our procedure in the same manner. Thus H'ase, which was clearly capable of degrading glycosaminoglycan ([^{3}H]-glucosamine labelled material) was unable to solubilise appreciable amounts of polypeptide ([^{3}H]-glycine labelled material) or glycopeptide ([^{3}H]-fucose labelled material) (refer to Fig. 5). The small amount of peptide/glycopeptide material released by H'ase may occur as a consequence

of its action, destroying matrix integrity and thus allowing some proteinaceous material to be extracted from the matrix during the course of the incubation period. The action of trypsin on matrices labelled with [^{3}H]-fucose confirms the findings of the original authors of this method—namely, that this enzyme readily digests all glycopeptide material [26,27]. We also observed that matrix material prepared from cultures maintained in the presence of [^{35}S]-Na_2SO_4 was mostly sensitive to trypsin digestion ($\pm$75% of total radioactivity released in 3 h), which is again consistent with our contention that H'ase degrades non- or poorly sulfated glycosaminoglycans; trypsin only, uncrossed-linked peptide and glycoconjugate material; and collagenase its own specific substrates. We appreciate that this procedure only reflects the relative proportions of the different classes of matrix macromolecules present in the material synthesised under the conditions described. However, this type of methodology has been utilised successfully to look for even quite subtle differences in matrices elaborated by cultured cells [26,27,31,32,35,36]. The validation of our modified method (namely, the use of H'ase) allowed us to develop a procedure to assess the major effects of EGF, and its close analogue TFG_α, on matrix synthesis by hBST cells. For example, the data obtained by use of testicular hyaluronidase in the first step of our analysis procedure of matrix-material radiolabelled with [^{3}H]-glucosamine suggested that the relative amount of non- or poorly sulphated GAG chains that were associated with the ECMs produced by cells in presence of EGF was elevated. We confirmed this observation, in relation to hyaluronate synthesis, by subsequent detailed GAG analysis using H'ase preparations from *Streptomyces hyaluronolyticus* that have been shown to be substrate specific [30].

We used specific antibodies to estimate the relative levels of some the matrix associated macromolecules present in ECMs prepared from cells maintained in the presence of [^{3}H]-glycine. This allowed us to make corrections for differences in the amount of matrix material synthesised by supplemented and non-supplemented cultures and express the data as a ratio of [^{3}S]/[^{3}H]. We are aware that in the extensively cross-linked ECMs produced by hBST in culture it is possible that complete free access to some epitopes did not occur; thus, the data may not be considered as quantitative. Prior digestion of matrices with H'ase to facilitate antibody access to epitopes did not alter the [^{35}S]/[^{3}H] ratios obtained without such pretreatment (Fig. 7). In spite of these considerations it was clear that the relative amounts of glycopeptide material produced by cultures supplemented with EGF were depressed, and furthermore the ratios of the amounts of type I and IV collagen in ECMs from supplemented as compared to control cultures were changed markedly.

Therefore, with the analytical procedures described in this communication it has been possible to assess the major effects of a normal breast tissue growth factor (EGF) on the elaboration of extracellular material by hBST cells in culture. We also observed that TGF_α addition to cultures mimicked all the effects of EGF, which is consistent with what is known about this growth factor and its role in neoplastic breast tissue. We feel that this in vitro model of ECM synthesis and its modulation by breast tissue growth factors (EGF and TGF_α) will be useful for the study of the process of tumour metastasis as it pertains to breast cancer. Similar studies have been reported using matrix material produced by cultured cells [for review see 35]; however, to our knowledge the use of matrices elaborated by human breast stromal cells has not as yet been reported. Also desmoplasia, "the tumour host-tissue response" [35,37,38,39], which plays a fundamental

role in the process of tumour invasion, can be studied using hBST cells in co-culture with tumour cells derived from biopsy material. Preliminary results using established breast tumour cell lines in co-culture with hBST have shown that there are marked differences in the degradation rates of matrices elaborated by the latter under the conditions (control vs. EGF) described here. We have not as yet established whether the differential rates of matrix degradation we have observed are as a consequence of enhanced production of degradative enzymes by the tumour cells or by the host (hBST) cells. This type of model system will allow us to answer some of these questions which are fundamental to our understanding of the process of tumour metastasis.

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