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Amphiregulin in lung branching morphogenesis: interaction with heparan sulfate proteoglycan modulates cell proliferation

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SUMMARY

Epithelial and mesenchymal cells isolated from mouse embryonic lungs synthesized and responded to amphiregulin (AR) in a different fashion. Mesenchymal cells produced and deposited 3- to 4-fold more AR than epithelial cells, proliferated in the presence of exogenous AR, and their spontaneous growth was blocked by up to 85% by anti-AR antibodies. In contrast, epithelial cells exhibited a broad response to this growth regulator factor depending on whether they were supplemented with extracellular matrix (ECM) and whether this ECM was of epithelial or mesenchymal origin. AR-treated epithelial cells proliferated by up to 3-fold in the presence of mesenchymal-deposited ECM, remained unchanged in the presence of epithelial-deposited ECM, and decreased in their proliferation rate below controls in the absence of ECM supplementation. This effect was abolished by treatment with the glycosaminoglycan-degrading enzymes heparinase and heparitinase suggesting the specific involvement of heparan sulfate proteoglycan (HSPG) in AR-mediated cell proliferation. In whole lung explants, branching morphogenesis was inhibited by antibodies against the AR heparan sulfate binding site and stimulated by exogenous AR. Since during development, epithelial cells are in contact with mesenchymal ECM at the tips of the growing buds and alongside the basement membrane, focal variations in the proportion of epithelial and mesenchymal HSPG will focally affect epithelial proliferation rates. Therefore, AR-HSPG interaction may underlie the process of branching morphogenesis by inducing differential cell proliferation.

Key words: amphiregulin, lung development, extracellular matrix, heparan sulfate proteoglycan, cell proliferation, branching morphogenesis, mouse

INTRODUCTION

The developing lung is composed of epithelial-lined airways surrounded by mesenchyme. During morphogenesis, the airways undergo a process of active growth and branching. This process is characterized by differences in the rate of epithelial cell proliferation depending upon their anatomical localization in the bronchial tree. Thus, cells localized in the proximal epithelium or within branching clefts divide at a significantly lower rate than those present in the tips of growing buds (Bernfield and Banerjee, 1972; Bernfield et al., 1972; Gallagher, 1986a). Focal and coordinated variations in cell proliferation result in the development of a branched airway tree. The mechanisms underlying this process are partially understood. Previous studies suggested that epithelial cell division during branching morphogenesis is determined by factors extrinsic to the cells. Among these are components of the extracellular matrix (ECM) (Bernfield et al., 1984, Minoo and King, 1994, Schuger et al., 1990, 1995), as well as mesenchymal-derived growth factors (Minoo and King, 1994; Rosen et al., 1994).

Recent studies indicate that mesenchymal control over epithelium may involve interaction between epidermal growth factor receptor and its ligands (Goldin et al., 1980; Schuger et al., 1993; Warburton et al., 1992). Amphiregulin (AR) is a recently characterized member of the epidermal growth factor family and interacts with cells through the epidermal growth factor receptor (Johnson et al., 1993a). AR is a 78-84 amino acid, glycosylated protein, initially synthesized as a 252-amino acid trans-membrane precursor (Shoyab et al., 1988, 1989) which migrates with a median relative molecular mass of approximately 20.7×10^3 in SDS-PAGE (Shoyab et al., 1988; Johnson et al., 1992). It was originally isolated from conditioned medium of the human breast carcinoma cell line MCF-7 (Shoyab et al., 1988). AR is expressed in multiple tissues and is evolutionary and structurally related to epidermal growth factor and transforming growth factor-α (Shoyab et al., 1989). Its major role is in the regulation of cell proliferation. AR promotes the growth of carcinoma cells, fibroblasts, normal mammary epithelial cells and keratinocytes (Cook et al., 1991; Li et al., 1992; Normanno et al., 1994; Pierkorn et al., 1994),

but inhibits the growth of some normal and neoplastic cell lines (Shoyab et al., 1988, Johnson et al., 1991). The ability to either stimulate or inhibit cell proliferation inspired the name 'amphiregulin' (Shoyab et al., 1988).

The present study employed recombinat AR, polyclonal antibodies against an AR synthetic peptides (Johnson et al., 1992), and a combination of lung epithelial and mesenchymal monocultures, cocultures and full lung explants to determine the presence and functional role of AR in the developing lung. AR was synthesized mostly by lung mesenchymal cells and served as an autocrine mitogen for the mesenchyme and as an autocrine/paracrine growth regulator for the epithelium. Unlike mesenchymal cells, lung epithelial cells responded to AR with either an increase or a decrease in proliferation, depending upon presence and origin of their surrounding ECM. Furthermore, AR-induced mitogenesis was abolished by glycosaminoglycan-degrading enzymes suggesting the involvement of heparan sulfate proteoglycan (HSPG) in the cell response to AR. Taken together, these studies suggest that interaction between different HSPGs and AR may control differential epithelial growth and thereby underlie the process of branching morphogenesis.

MATERIALS AND METHODS

Lung organ cultures

These were generated as previously described (Schuger et al., 1990a). CD-1 strain (Charles River) mice were mated and the day of finding a vaginal plug was designated as day zero of embryonic development. Embryos were collected at days 12 and 13 of gestation. Their lungs were then dissected and the lower right lobes were cultured at the air-medium interface on the upper surface of polycarbonate filter membrane inserts, (Millipore, Bedford, MA) in minimal essential medium (MEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), (Hyclone Lab., Logan, UT), nonessential amino acids (Gibco), L-glutamine, and antibiotics (Irvine Scientific, Santa Ana, CA). FBS was omitted in some experiments and in others a serum-free defined medium, BGJb (Gibco), was used instead.

Epithelial and mesenchymal cell monocultures and cocultures

Monocultures were generated from day-15 to -16 lungs by differential plating as previously described (Schuger et al., 1993). Culture samples were immunostained with an anti-keratin antibody to identify epithelial cells. Only mesenchymal monocultures with less than 1% keratin-positive cells and epithelial monocultures with 10% or less mesenchymal cell contamination were used for experimentation. Epithelial-mesenchymal cocultures were generated by adding epithelial cells to a confluent or semi-confluent mesenchymal monocultures, or by plating mixed lung cell populations isolated from fetal lungs.

AR and antibodies to AR

Human recombinant AR (Pierkorn et al., 1994) was used for these studies. Two rabbit polyclonal antibodies, AR-Ab1 and AR-Ab2, were generated against synthetic peptides extending from amino acids 8 to 26 (AR-Ab1) and from amino acids 26 to 44 (AR-Ab2) of the AR molecule. These regions are highly homologous between human and mouse AR (Kimura et al., 1990). Immunoglobulin G (IgG) from AR-Ab1 as well as from preimmune rabbit serum were purified by protein-G agarose and AR-Ab2 was affinity purified against the immobilized peptide (Johnson et al., 1993b). AR-Ab-2 is directed against the region of AR which binds to heparin (Johnson and Wong, 1994). Antibodies AR-Ab1 and AR-Ab2 detect the human and murine AR protein in immunoprecipitation, western blotting, ELISA and immunohistochemical applications (Johnson et al., 1993b; Kenney et al., 1995). The antibodies exhibit no cross-reactivity with epidermal growth factor, transforming growth factor-α (Johnson et al. 1992), or heparin-binding epidermal growth factor-like growth factor (G. R. Johnson, unpublished observations).

Metabolic labeling, immunoprecipitation and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Lungs from embryos at days 12 and 13 of gestation, and mesenchymal and epithelial cell monocultures were incubated overnight in methionine-free medium supplemented with 100 mCi/ml of [^{35}S]methionine (1.0-1.4 Ci/mmol; NEN-Dupont, Boston, MA). After incubation, the tissues and cells were lysed and centrifugated at 10,000 ***g*** for 10 minutes. AR was precipitated from the lysates with 2 μg/ml of AR-Ab1 or AR-Ab2 antibody and protein A-Sepharose (Sigma, St. Louis, MO) according to the protocol of Ruddon et al. (1979). The immunoprecipitates were eluted and fractionated in a 15% polyacrylamide gel. Radioactive bands were visualized by exposing the dried gels to X-ray film (Kodak XAR-2, Eastman Kodak Co., Rochester, NY).

Enzyme linked immunosorbent assay (ELISA)

Epithelial and mesenchymal monocultures were incubated for 3 hours in serum-free MEM containing 0.02% bovine serum albumin (BSA), (MEM-BSA). The culture fluids were then collected and added to wells of a 96-well plate (Falcon Plastics, Oxnard, CA) in aliquots of 0.1 ml/well. MEM-BSA served as a negative control. Serial concentrations of AR were added to the assay plates to serve as standards. After the 4-hour incubation, the fluids were removed and ELISA was performed as described by Varani et al. (1985) using either AR-Ab1 or AR-Ab2 antibodies.

Immunohistochemistry

Mesenchymal cell monolayers were fixed in absolute alcohol for 2 minutes and then incubated with 8 μg/ml of antibodies AR-Ab1, AR-Ab2 or preimmune IgG for 45 minutes at room temperature. The sections were then washed and immunostained using a commercial peroxidase-anti-peroxidase kit (Dako, Carpinteria, CA), following the manufacturer's instructions. As an additional control, sections were treated with PBS containing no primary immunoglobulin.

In order to identify epithelial cells in cocultures these were fixed in absolute alcohol for 2 minutes and immunostained with a rabbit polyclonal antibody to cytokeratins of low and high molecular mass using a commercial peroxidase-anti-peroxidase kit (both from Dako). The effectiveness of this procedure in identifying lung fetal epithelial cells has been previously reported (Schuger et al., 1993).

Epithelial and mesenchymal-deposited ECM

Confluent epithelial and mesenchymal cell monocultures established in 8-chamber slides (Nunc Inc., Naperville, IL) were treated with 0.5 mM EDTA or 2 M urea in MEM (the latter containing 0.5% FBS) for 30 minutes at 37ºC. After cells had retracted, they were removed from their substrate by gently adding 1 ml of the same solution several times and aspirating it. Treatment with 2 M urea has been extensively used to remove cells with minimal or no cell lysis, leaving behind an intact matrix (Gospodarowicz et al., 1983; Vlodavsky et al., 1990). The amount of matrix deposited by the cells was determined in parallel 96-well plates using a microtiter assay for protein quantitation (Pierce, Rockford, IL). The deposited ECM was used directly for functional studies, or otherwise extracted for binding assays. Matrix was extracted by adding to the dishes 1% SDS in PBS and warming for 10 minutes at 90°C. The detergent was removed by running the samples through an AG 11 A8 resin column (Bio Rad, Hercules, CA). The samples were concentrated and their protein content was deter-

mined. Laminin, fibronectin and thrombospondin were detected in this matrix extract by immunobloting (not shown).

Radio-iodination of AR

Recombinant AR (100 μg) was dissolved at 1 μg/ml in PBS and placed in a vial with 10 μg of Iodogen (1,3,4,6-tetrachloro-3a-6a-diphenylglycouril; Sigma). Radioactivity was added and the reaction allowed to proceed for 15 minutes at room temperature. Labeled protein was separated from unreacted ^{125}I by chromatography on a Sephadex G-25 column. Incorporation of ^{125}I was assessed by precipitation of AR on filter paper with 10% trichloroacetic acid. Initial specific activity was approximately 1 mCi/mg.

Binding assays

Quadruplicate wells of a 96-well plate were coated with various concentrations of epithelia-derived and mesenchyme-derived ECM, with matrix directely deposited by these cells, or used as a bare plastic surface. The plates were washed and incubated for one hour at 4°C in serum-free medium with the addition of 0.1% BSA and approximately 10^5 cpm of ^{125}I-AR. At the end of this incubation the wells were washed, 1% SDS was added to each well and the plate was placed at 90°C for 5 minutes to dissolve the various matrices. These were collected and the amount of radioactive AR bound to each well was determined in a γ-counter. In additional studies, the matrices were treated with 0.02 units/ml of heparinase, heparitinase or chondroitinase (Sigma) for 18 hours and washed prior to conducting the AR binding assays. Nonspecific binding was assessed in parallel cultures which received a 200-fold excess of unlabeled AR. Nonspecific binding was 10-15% of the total binding and was subtracted from all points.

ELISA was used as an additional means to determine the amount of AR bound to the various matrices, as well as to determine the amount of native AR present in the epithelial and mesenchymal cell-derived matrix.

Proliferation assays in epithelial and mesenchymal monocultures and cocultures

Epithelial and mesenchymal monocultures and cocultures were established in 8-chamber slides with or without a previously deposited epithelial or mesenchymal ECM. Cells were plated at a density of 1-2×10^4 cells/chamber and left to attach overnight. The next day, the wells were washed and incubated for 48 hours in the presence of AR (at concentrations of 2.5-50 nM), antibodies AR-Ab1, AR-Ab2, IgG (each 10 to 50 μg/ml), 10 nM AR preincubated with 10 μg/ml of antibody AR-Ab2, or left untreated. Subsequently the attached cells were trypsinized, counted in a Coulter counter, and the number of cells in the AR-, AR-Ab2-, AR/AR-Ab2-, and IgG-treated and untreated chambers was compared. Epithelial-mesenchymal cocultures were immunostained with anti-keratin antibodies, counterstained with hematoxylin, and the keratin-positive cells (epithelial) were counted on a projection screen. Ten fields were quantified in each chamber to produce an average number per chamber.

Tritiated thymidine incorporation and autoradiography were used as an additional means to assess cell proliferation. One μCi of [^{3}H]thymidine per 0.2 ml was added to semi-confluent epithelial and mesenchymal monocultures established in 8-chamber slides. After 4 and 24 hours, the cultures were fixed in absolute alcohol, dipped in NTB2 emulsion (Eastman Kodak Co., Rochester, NY), and exposed for 48 hours at 4°C. Autoradiographs were then developed and the percentage of labeled nuclei was determined on a projection screen. Ten fields were counted in each chamber to obtain an average number per chamber. All the experiments presented here were run in quadruplicate chambers and repeated at least four times. The Student's *t*-test was used to determine statistical significance of the results.

Branching morphogenesis in lung organ culture

Day-12 and -13 lung explants established as organ cultures were exposed for 48 hours to AR at doses of 5, 10 and 15 nM, and 10 nM AR preincubated with 10 μg/ml of antibody AR-Ab2, in the presence of 10% FBS, in serumless MEM and in a defined serumless medium (BJGb). At the end of this period the number of airway terminal branches was determined and compared to untreated explants. Blocking experiments using anti-AR antibody were carried out in an attempt to determine whether lung endogenous AR was acting as an autocrine/paracrine factor in this organ. Lung organ cultures were exposed to 50 or 100 μg/ml of antibodies AR-Ab1, AR-Ab2 or control rabbit IgG. Branching activity was determined after 48 hours and compared.

Culture exposure to heparinase, heparitinase and chondroitinase

Semi-confluent epithelial cell monocultures established on mesenchymal-deposited ECM as well as semi-confluent mesenchymal monocultures were exposed to 5 nM AR with or without 0.02 units/ml of heparinase, heparitinase or chondroitinase. Enzymes were added at time zero and after 24 hours. Cultures were incubated for 48 hours, trypsinized and the number of cells was determined. In additional studies, the mesenchymal-deposited ECM was treated with the enzymes for 2 hours prior to plating the epithelial cells. The enzymes were removed by extensive washing prior to plating the epithelial cells, these were cultured for 24 hours and then exposed to 1 μCi of [^{3}H]thymidine/ml. After 6 hours the cultures were fixed in absolute alcohol, dipped in NTB2 emulsion (Eastman Kodak Co., Rochester, NY), and exposed for 48 hours at 4°C. Autoradiographs were then developed and the percentage of labeled nuclei was determined on a projection screen. Ten fields were counted in each chamber to obtain an average number per chamber.

RESULTS

Mouse embryonic lungs synthesize AR

AR was immunodetected in lung cells (Fig. 1). Antibodies AR-Ab1 and AR-Ab2 immunoprecipitated a single major band of approximately 20,000 M_r protein from whole mouse lungs and from cell monolayers, consistent with AR (Fig. 1). This band was not observed when rabbit IgG was substituted for anti-AR

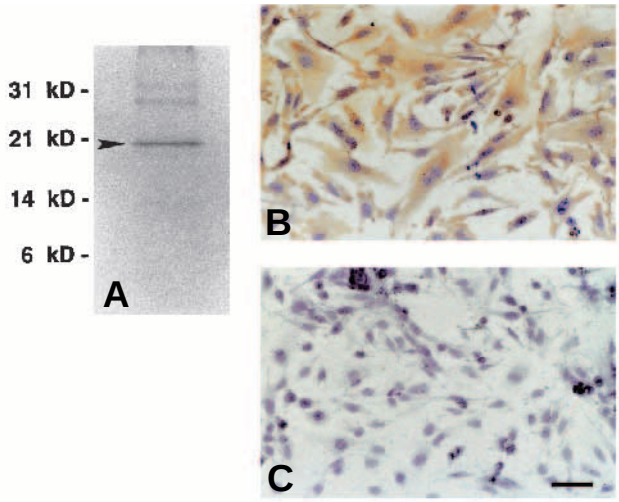

Fig. 1. Immunodetection of AR in lung cells. (A) The arrow points to AR immunoprecipitated from day-12 lungs. Two additional bands of weaker intensity are seen migrating with a M_r of approximately 28-35×10^3. Since AR is cleaved from a precursor protein, these may represent additional isoforms of larger molecular mass. (B) Lung mesenchymal cells immunostained with antibody AR-Ab1 show a light brown color in the cytoplasm. No immunoreaction is seen with preimmune IgG (C).

Table 1. Production of soluble AR by epithelial and mesenchymal monocultures

Cell type (10^5)	AR production (pg/3 hours) Mean	Range*
Mesenchymal	38.800±36	(2.4-75)
Epithelial	10.900±28	(0.24-43)

*The high ranges are due to pronounced differences between experiments however in each of them the mesenchymal/epithelial ratio for AR production consistently ranged from 2 to 10.

antibody (not shown). Two additional bands of weaker intensity were seen migrating with a M_r between 28,000 and 35,000. ELISA further confirmed that AR was produced by both epithelial and mesenchymal cells, the two main cell populations of the embryonic lung. However, monocultures of mesenchymal cells produced 3- to 4-fold more AR per cell than monocultures of epithelial cells (Table 1).

AR and antibodies to AR respectively stimulated and inhibited branching morphogenesis

Embryonic lung explants exposed to various doses of AR had a statistically significant increase in the number of terminal airway buds compared to untreated explants. This increment was dose-dependent, with 50% and 100% increases in the number of branches for concentrations of 5 and 10 nM, respectively (Fig. 2). Lung explants exposed to 50 μg/ml of antibody AR-Ab2, but not to the same concentration of antibody AR-Ab1 (not shown) or preimmune IgG, exhibited a decrease in the number of terminal airway buds after 2 days in culture (Fig. 2).

AR stimulated mesenchymal cell proliferation and inhibited epithelial cell proliferation in lung cell monocultures

Mesenchymal cell monocultures exposed to various concentrations of AR showed a statistically significant increase in the number of cells after 48 hours as compared to untreated controls (Fig. 3). This stimulation was independent of the presence or absence of FBS. AR-Ab2 at concentrations of 10 μg/ml blocked mesenchymal cell proliferation suggesting that AR functions as an autocrine growth stimulator for mesenchymal cells in monoculture (Fig. 3). Antibody AR-Ab1 had no effect on cell proliferation (not shown). These results were confirmed by [^{3}H]thymidine incorporation assays which showed an average of 92±24 labeled nuclei/chamber in monocultures exposed to 5 nM AR compared to 20±12 labeled nuclei/chamber in controls.

Epithelial cells established as primary monocultures showed a concentration-dependent inhibition in proliferation in the presence of AR (whether in medium with or without FBS; Fig. 4). These results were confirmed by [^{3}H]thymidine incorporation, which showed 6±3 labeled nuclei/chamber in monocultures exposed to 5 nM AR compared to 24±8 labeled nuclei/chamber in untreated controls. A possible cytotoxic effect of AR was ruled out by a ^{51}Cr release assay (Varani et al., 1985) which showed no difference in ^{51}Cr release between epithelial cells exposed to 10 nM AR and unexposed cells, and by abrogating the effects of 10 nM AR by a preincubation with 10 μg/ml of AR-Ab2 (data not shown). Epithelial cells exposed to AR antibodies essentially behaved as the controls (Fig. 4).

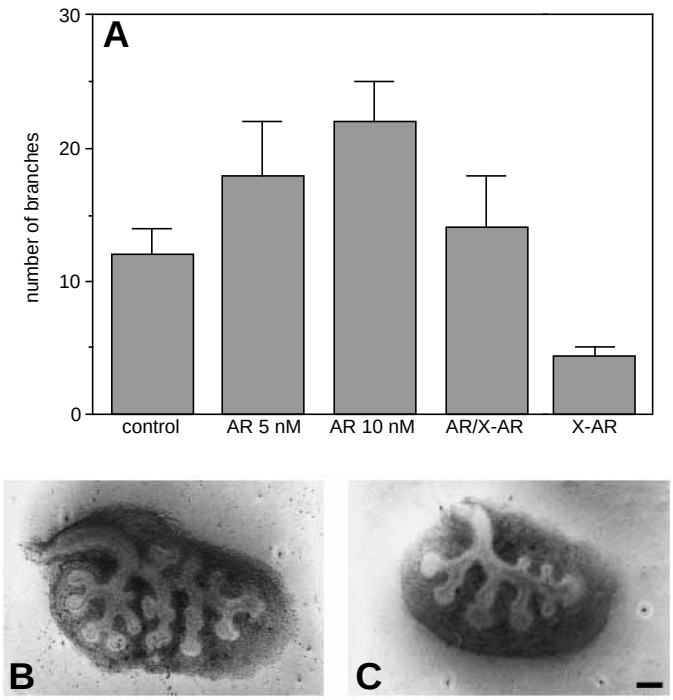

Fig. 2. Effect of AR on branching morphogenesis. (A) Lung explants from day-13 mouse embryos were exposed to, 5 nM AR, 10 nM AR preincubated with 10 μg/ml of antibody AR-Ab2 (AR/X-AR), antibody AR-Ab2 (50 μg/ml; X-AR) or rabbit IgG (control). The extent of branching activity was determined after 48 hours by direct counting of terminal buds in each explant. Bars represent standard deviation. Both stimulation of branching activity with 10 nM AR and inhibition with 50 μg/ml of anti-AR were statistically significant (P<0.01). (B,C) Lung explants from day 12 of gestation after 24 hours in serum-free minimal essential medium with (B) and without 10 nM AR (C). Scale bar, 100 μm.

AR stimulated epithelial cell proliferation in the presence of mesenchymal cells or mesenchymal ECM

The inhibitory effect of AR on epithelial cell proliferation was reversed by culturing them in combination with mesenchymal cells (coculture; Fig. 5), or in dishes in which an ECM was previously deposited by confluent mesenchymal cells (Fig. 6A). The magnitude of the proliferative response induced by AR did not change significantly within the range of matrix quantities deposited (varying from approximately 10 to 20 μg matrix/chamber). These results were confirmed by [^{3}H]thymidine incorporation, which showed 72±8 labeled nuclei/chamber in monocultures exposed to 5 nM AR in the presence of mesenchymal-derived matrix compared to 22±6 labeled nuclei/chamber in monocultures grown in the same matrix but unexposed to AR.

In contrast to what was observed with mesenchymal-deposited ECM, the inhibitory effect of AR on epithelial cell proliferation was not reversed by epithelial-deposited ECM. The anti-mitogenic effect of AR was however neutralized by culturing epithelial cells on epithelial-deposited ECM (Fig. 6B). Epithelial cells did not proliferate at statistically higher

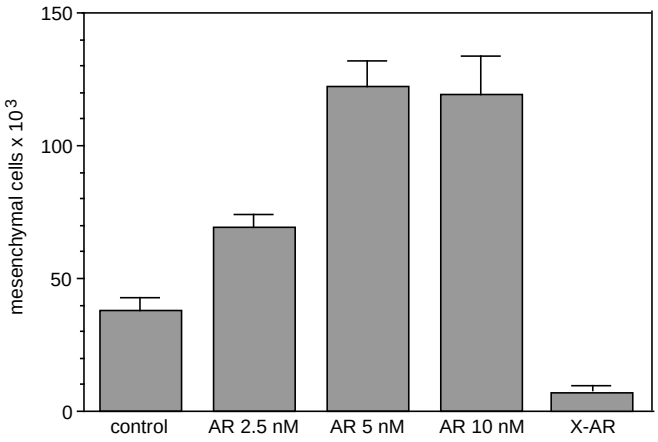

Fig. 3. Effect of AR on lung mesenchymal cell proliferation. Mesenchymal cell monocultures were exposed to various concentrations of AR, antibody AR-Ab2 (10 μg/ml) or rabbit IgG (control). The number of cells per chamber was determined after 48 hours. The differences in cell number between monocultures treated with AR and antibody AR-Ab2 were statistically significant compared to control monocultures (P<0.01 or lower).

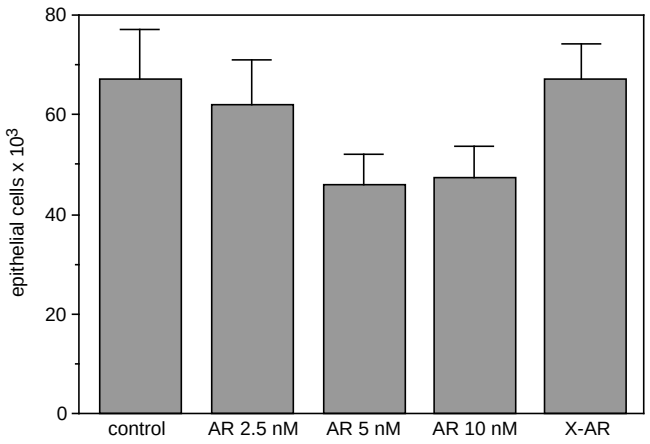

Fig. 4. Effect of AR on lung epithelial cell proliferation. Epithelial cell monocultures were exposed to AR, antibody AR-Ab2 (10 μg/ml), or rabbit IgG (control). The number of cells per chamber was determined after 48 hours. The differences in cell number between treated and control monocultures were statistically significant for AR concentration of 5 and 10 nM (P<0.05).

rate than those not exposed to AR, despite the fact that the amounts of epithelial and mesenchymal-deposited ECM were in general similar (up to 20 μg/well).

Epithelial and mesenchymal-derived ECM bound similar concentration of exogenous AR, but mesenchymal-derived ECM was richer in endogenous AR

These studies were conducted to determine whether AR binds to epithelial-derived ECM and whether the differences in cell proliferation observed in the previous experiments could be related to quantitative differences in AR binding. No statistically significant differences were observed between the two types of matrix, whether directly deposited by the cells (not shown) or extracted (Fig. 7A). Treatment of the matrices with heparinase, but not with chondroitinase (not shown), abrogated AR binding to both matrices to a similar extent (Fig. 7A). Likewise, incubation of AR with antibody AR-Ab2 inhibited binding (Fig. 7B). ELISA showed that mesenchymal-derived ECM contains approximately 4- to 5-fold more AR than epithelial-derived ECM (Table 2), which may represent a result of higher mesenchymal production. These differences were abolished by immunoprecipitating and removing the AR from the matrix extracts prior to carrying out the ELISAs, which then showed only traces of AR.

Treatment with heparinase and heparitinase abolished AR-induced cell proliferation

Epithelial cell monocultures established on ECM deposited by mesenchymal cells and as well as mesenchymal cell monocultures decreased their proliferation rate by approximately 3 and 10-fold respectively when exposed to AR in the presence of heparinase or heparitinase. A possible cytotoxic effect of the heparan sulfate-degrading enzymes was ruled out by a ^{51}Cr release assay (Varani et al., 1985). This showed no difference in ^{51}Cr release between epithelial cells exposed and unexposed to heparinase or heparitinase and by abrogating the effects of 10 nM AR by a preincubation with 10 μg/ml of AR-Ab2 (data not shown). Exposure to chondroitinase did not affect AR-induced cell proliferation (Fig. 8). Pretreatment of mesenchymal-derived matrix with heparinase or heparitinase, but not chondroitinase, resulted in a similar inhibition of epithelial cell proliferation: 54±12 labeled nuclei/chamber in monocultures exposed to 5 nM AR in the presence of untreated ECM compared to 14±3 labeled nuclei/chamber in monocultures exposed to 5 nM AR in the presence of enzyme-treated matrix.

Table 2. Detection of endogenously produced AR in epithelial and mesenchymal-derived ECM

Matrix (μg)	AR (pg)
Epithelial	
10	10±30
50	45±20
Mesenchymal	
10	50±20
50	230±85

DISCUSSION

Differential rates of cell division along the bronchial tree are essential for the process of branching morphogenesis. There is increasing evidence indicating that components of the ECM/basement membrane as well as mesenchymal growth factors influence epithelial branching (Minoo and King, 1994). Previous studies suggested that mesenchymal control of epithelial proliferation is in part mediated by the interaction of epidermal growth factor receptor and one or more of its ligands (Goldin et al., 1980; Warburton et al., 1992; Schuger et al., 1993). The present study indicates that AR, a member of the epidermal growth factor family, may be one of the ligands involved in this pathway.

Epithelial and mesenchymal cells isolated from mouse embryonic lungs synthesize and respond differently to AR

Mesenchymal cells synthesized approximately 3- to 4-fold

more AR than epithelial cells and used AR as an autocrine mitogenic factor, as indicated by inhibition of autonomous mesenchymal cell proliferation by anti-AR antibodies. Since exogenous AR stimulated mitogenesis by an additional 3-fold, this effect, added to the effect of endogenous AR, indicated an approximately 10-fold increase in cell proliferation upon maximal AR stimulation. Although AR has been shown to be mitogenic for mature fibroblasts (Shoyab et al., 1988) as well as having an autocrine function for several epithelial cell lines (Cook et al., 1991; Li et al., 1992; Normanno et al., 1994), there was no previous evidence indicating an autocrine function in cells of mesenchymal lineage.

The higher levels of AR production by mesenchymal cells were correlated with an increased deposition in the mesenchymal ECM. Although epithelial- and mesenchymal-derived matrices bound similar quantities of radiolabeled AR, their respective binding affinities for AR were not determined. Therefore the possibility that AR remains associated with the mesenchymal ECM for longer periods of time, resulting in higher levels of mesenchymal AR, was not ruled out.

Lung epithelial cells exhibited a wide response to AR which ranged from a moderate but consistent decrease in proliferation to an approximately 3-fold stimulation over a period of 48 hours. The type of response depended on whether the cultures were supplemented with ECM and whether this ECM was of epithelial or mesenchymal origin. AR-treated epithelial cells proliferated in the presence of mesenchymal-deposited ECM, remained unchanged in the presence of epithelial-deposited ECM, and decreased in their proliferation rate below controls in the absence of matrix supplementation. These findings suggest that AR in combination with different concentrations and types of ECM can direct embryonic epithelial cells to either proliferate or stop proliferating. In whole lung explants this effect resulted in stimulation of branching morphogenesis as demonstrated here.

HSPG is involved in AR-induced mitogenesis

Studies on colon carcinoma cell lines as well as on human keratinocytes suggested that autocrine and paracrine signaling by AR may require cellular heparan sulfate proteoglycan (HSPG), presumably as matrix or membrane proteoglycans (Cook et al., 1991; Li et al., 1992; Piepkorn et al.,

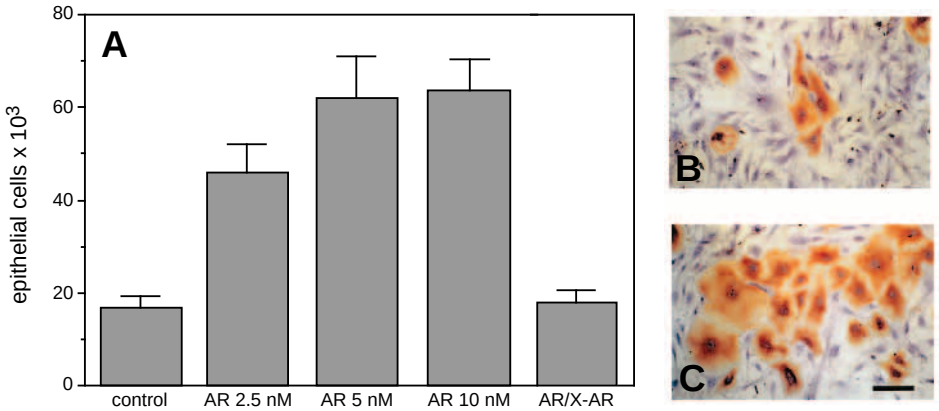

Fig. 5. Effect of AR on lung epithelial cell proliferation in the presence of mesenchymal cells. (A) Epithelial-mesenchymal cocultures were exposed to, AR, 10 nM AR preincubated with 10 μg/ml of antibody AR-Ab2 (AR/X-AR), or rabbit IgG (control). The number of cells per chamber was determined after 48 hours. The differences in epithelial cell number between cocultures treated with AR versus controls and cocultures treated with AR/AR-Ab2 were statistically significant (P<0.01 or lower). (B,C) Photomicrographs of cocultures exposed to 5 nM AR (B) and unexposed control (C). The epithelial cells are stained brown and the mesenchymal cells blue. Scale bar, 20 μm.

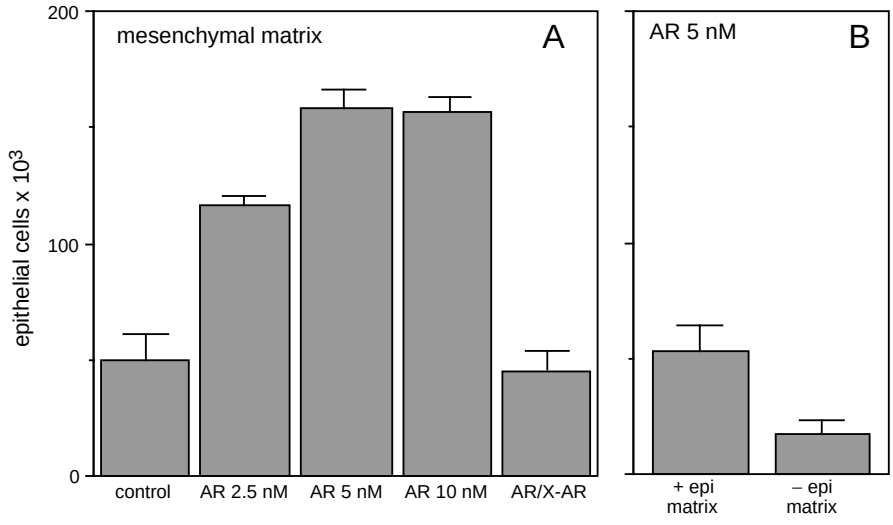

Fig. 6. (A) Effect of AR on lung epithelial cell proliferation in the presence of mesenchymal-deposited ECM. Epithelial monocultures established in chambers coated with mesenchymal ECM were exposed to, AR, 10 nM AR preincubated with 10 μg/ml antibody AE-Ab2 (AR/X-AR) or left untreated (control). The number of cells per well was determined after 48 hours. The differences in cell number between AR-treated versus AR/AR-Ab2 treated and control monocultures were statistically significant (P<0.01 or lower). (B) Effect of AR on lung epithelial cell proliferation in the presence of epithelial-deposited ECM. Epithelial monocultures established in chambers coated or uncoated with epithelial ECM (+ epi matrix and – epi matrix, respectively) were exposed to AR. The number of cells per chamber was determined after 48 hours. The differences in cell number between treated and control were statistically significant (P<0.05).

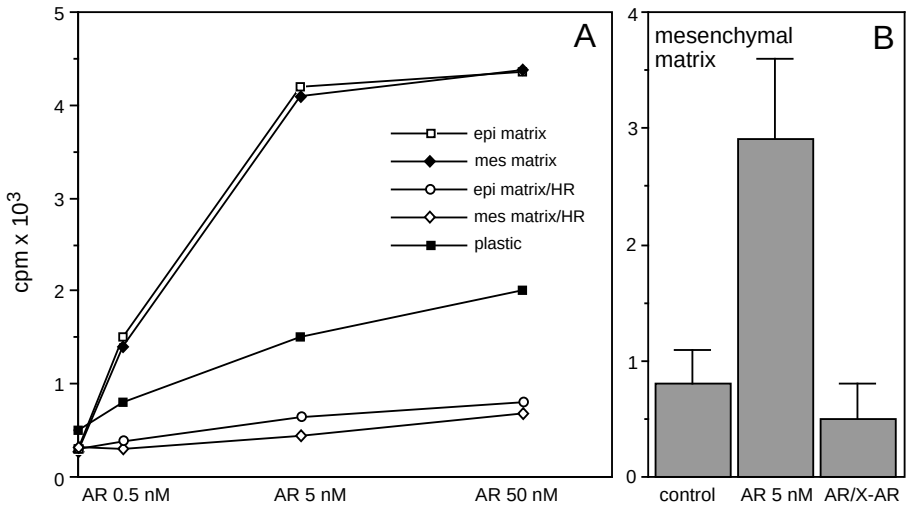

Fig. 7. (A) AR binding to the ECM. Uncoated wells and wells coated with 10 μg of epithelial- or mesenchymal-derived ECM, with or without treatment with 0.02 U/ml of heparinase, were incubated with ^{125}I-AR. Radioactive binding was determined 1 hour later. AR binding to epithelial- and mesenchymal-derived ECM was similar and significantly higher than to uncoated plastic (P<0.05 or lower). AR binding to both matrices was abrogated by preincubating them with heparinase (HR). Standard deviations were below 10% of the total values and were not included in the graph. (B) AR binding to mesenchymal-derived ECM was blocked by preincubation with 10 μg/ml of antibody AR-Ab2 (AR/X-AR). Untreated epithelial cell monocultures established on mesenchymal-derived ECM were used as controls.

1994). However, direct evidence for the role of heparan sulfate in AR-induced mitogenesis has been presented only recently (Johnson and Wong, 1994). In accordance with these previous studies, when fetal lung epithelial and mesenchymal cells were exposed to glycosaminoglycan-degrading enzymes, heparinase and heparitinase, the ability of AR to evoke mitogenesis was lost. Furthermore, antibody AR-Ab2, which binds to the AR heparan sulfate-binding region (Johnson and Wong, 1994), but not antibody AR-Ab1, inhibited mesenchymal cell growth and branching morphogenesis. These results strongly suggested that HSPG is involved in AR-induced lung morphogenesis.

Quantitative or qualitative differences in HSPG could explain the different effect of mesenchymal and epithelial ECM on AR-induced mitogenesis. Although there is no information regarding whether mesenchymal cells produce more HSPG than epithelial cells, there is evidence indicating that HSPG composition of epithelium and mesenchyme are different. For example, syndecan-1, a major cell surface HSPG, is mainly expressed in the epithelium of developing lungs (Brauker et al., 1991; David et al., 1993), whereas syndecan-2 is expressed in the mesenchymal cells surrounding the epithelial stalks and branches (David et al., 1993). Moreover, a monoclonal antibody against an as yet uncharacterized HSPG(s) immunostains only the mesenchymal cells that confront the growing epithelial tips (David et al., 1992). Therefore, although AR seems to bind to heparan sulfate chains of epithelial and mesenchymal proteoglycans (as shown by our studies), AR may have a functional interaction only with HSPG(s) found mainly in the mesenchyme. In support of such a possibility, recent studies demonstrated that basic fibroblast growth factor binds similarly to HSPGs syndecan-1, syndecan-2, glypican and perlecan, but only the latter promotes basic fibroblast growth factor-induced mitogenesis (Aviezer et al., 1994a,b).

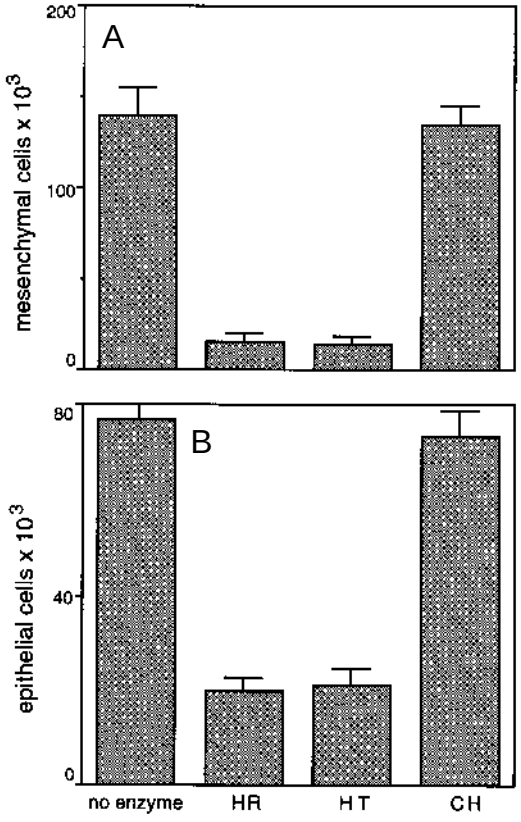

Fig. 8. (A) Mesenchymal cell monoculture exposed to AR with or without 0.02 U/ml of heparinase (HR), heparitinase (HT) or chondroitinase (CH) and cultured for 48 hours. (B) Epithelial cell monoculture established on mesenchymal-deposited ECM and exposed to 10 nM AR with or without the various enzymes as in A. The differences in cell number between HR- and HT-treated cultures were statistically significant compared to cultures treated with CH or AR alone (P<0.01 or lower).

Clues for a mechanism leading to branching morphogenesis

By integrating the action of AR and the ECM, these studies provide a basis to explain the differential rates of epithelial cell proliferation occurring during branching morphogenesis. First, our studies showed that AR produced maximal epithelial cell proliferation in the presence of mesenchymal ECM. During lung morphogenesis, epithelial cells are in direct contact with the mesenchyme only at the tips of growing bronchial buds. This contact is possible because of discontinuities in the BM (Bluemink et al., 1976; Grant et al., 1983; Jaskoll and Slavkin, 1984) through which epithelial cells extend multiple cytoplas-

mic protrusions (Bluemink et al., 1976; Jaskoll and Slavkin, 1984). This unique circumstance places the distal most epithelium in close contact with an essentially pure mesenchymal ECM, which is also relatively rich in AR. Such combination of events could explain the higher proliferation rate observed at the tips of the growing buds.

Second, our studies showed that epithelial cells growing on epithelial instead of mesenchymal-deposited ECM did not proliferate over baseline rates upon AR stimulation. A similar microenvironment is found in vivo alongside the trachea and proximal segments of the bronchial tree, where a well developed basement membrane is found (Bernfield et al., 1984; Gallagher, 1986b, Jaskoll and Slavkin, 1984). Therefore, interaction of AR with an epithelial-derived basement membrane may explain the relatively lower epithelial proliferation rate observed in the proximal segments of the bronchial tree. In addition, since the basement membrane has a dual epithelial and mesenchymal origin (Loreal et al., 1993; Sariola et al., 1984; Simon-Assmann et al., 1988; Skinner et al., 1985), the relative contribution of epithelial and mesenchymal cells to the basement membrane may further regulate epithelial cell proliferation.

Third, AR inhibited cell proliferation when added to epithelial monocultures without matrix supplementation. At present it is difficult to extrapolate this observation to the in vivo scenario. However, it should be noted that the apical surface of epithelial cells is relatively devoid of ECM. Perhaps release of AR to the luminal surface and/or into the lumen itself helps to prevent haphazard cell growth towards the bronchial lumen.

In summary, the mechanism suggested here provides a paradigm of how a growth regulator factor may elicit a range of proliferative cell responses depending upon the ECM. Since the developing basement membrane is a dynamic ECM structure of dual epithelial and mesenchymal origin, the restricted changes in its architecture and composition occurring during development may affect the epithelial cell response to AR. This, in turn, could contribute to the process of branching morphogenesis by focally modulating epithelial cell proliferation.

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