Pulmonary fibroblasts stimulate the proliferation of cell lines from human lung adenocarcinomas

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Human lung cancer cell lines are widely used to test anticancer drugs. These in-vitro tests, however, preclude the detection of responses to paracrine factors from surrounding stroma. We have cocultured pulmonary fibroblasts CCD-19Lu, from a healthy donor, or HLF-A, from a patient with epidermoid carcinoma of the lung, with two human pulmonary adenocarcinoma cell lines to test the hypothesis that the fibroblasts stimulate the growth of the tumor cells. Both fibroblast cell lines significantly increased the proliferation of the pulmonary adenocarcinoma cell lines in 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assays, with HLF-A fibroblasts yielding the most pronounced responses. The proliferation of the pulmonary adenocarcinoma cell lines in coculture with fibroblasts was blocked by antibodies against the transforming growth factor-α and amphiregulin. In addition, reverse transcription-polymerase chain reaction showed expression of mRNA for amphiregulin and transforming growth factor-α in all cell lines, whereas mRNA for the epidermal growth factor was detected only in pulmonary adenocarcinoma cell lines. Western blot analysis revealed that medium containing growth factors released by each fibroblast cell line activated extracellular signal-regulated kinase 1/2 in the both tested pulmonary adenocarcinoma cell lines, but activated Akt kinase only in A549 cells. Assessment of protein levels for cyclin D1 and cyclin E by Western blots demonstrated pronounced increases of both proteins in each pulmonary adenocarcinoma cell line, whereas protein levels for cyclin-dependent kinase inhibitor p21 remained unchanged. Immunocytochemical analysis showed positive immunoreactivity for P-extracellular signal-regulated kinase 1/2, cyclin D1 and cyclin E in pulmonary adenocarcinoma cells cocultured with fibroblasts or exposed to fibroblast-conditioned media. Our data suggest that the growth of pulmonary adenocarcinoma is stimulated by amphiregulin and transforming growth factor-α released from pulmonary fibroblasts. This may contribute to the disappointing clinical responses to anticancer drugs, which have shown promise in tests with lung cancer cell lines. *Anti-Cancer Drugs* **17:771–781**

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Introduction

Pulmonary adenocarcinoma (PAC) is the leading type of lung cancer today and demonstrates a mortality rate of above 85% within 5 years of diagnosis [1]. The absence of methods for the early detection of this malignancy and its general resistance to conventional therapeutics contribute to the poor prognosis.

Cell lines derived from different histological lung cancers in human patients have been used extensively during the past two decades for the identification of novel lung cancer therapeutics [2,3]. The effects of potential anticancer agents on the proliferation, apoptosis and viability of lung cancer cells are initially tested *in vitro*. Agents that yield promising results in the lung cancer cell lines are subsequently tested in mouse xenographs from such cell lines before going on into systemic toxicity testing in laboratory animals and, finally, clinical trials in human cancer patients. An enormous number of agents has thus been tested, many of which showed great promise *in vitro* and in the mouse xenographs. Unfortunately, to date, not a single drug has generated significant lasting clinical responses in PAC patients [1].

Many lung cancer cells produce growth factors, which provide an autocrine loop for the continuous stimulation of cancer growth [4]. In-vitro tests with lung cancer cell lines and in-vivo tests with mouse xenographs from such cell lines allow for the detection of agents that block this autocrine stimulation. Lung cancer cells, however, are additionally subjected to numerous factors in their immediate intrapulmonary environment that can modulate their responsiveness to anticancer drugs. In particular, surrounding stroma cells can release growth

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factors, extracellular matrix proteins and angiogenic factors [5–10], which may promote tumor growth and decrease responsiveness to therapeutics.

In the current study, we have cocultured the human PAC cell lines NCI-H322 and A549 with human fibroblast cell lines CCD-19, from a healthy donor, or HLF-A, from a patient with epidermoid carcinoma of the lung, to assess growth-modulating effects of the fibroblasts on the tumor cells. Our data show that both fibroblast cell lines significantly stimulated the proliferation of both PAC cell lines, a response inhibited by neutralization of the growth factors with antibodies against transforming growth factor (TGF)-α and amphiregulin (AR). These effects of fibroblasts on PAC cell lines involved the activation of extracellular signal-regulated kinase (ERK) 1/2 kinases, cyclin D1 and cyclin E in both PAC cell lines and the additional activation of phosphatidylinositol 3′-kinase/Akt kinase in A549 cells.

Materials and methods

Cell lines

All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and maintained in an atmosphere of 5% CO_2 at 37°C in media suggested by the supplier without antibiotics. The human PAC cells NCI-H322, which express phenotypic and functional features of bronchiolar Clara cells, were grown in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS). The human PAC cells A549, which express phenotypic and functional features of alveolar type II cells, were grown in Ham's F-12 K modification medium supplemented with 10% FBS. The human pulmonary fibroblast cell line derived from a healthy patient, CCD-19Lu, was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and the human pulmonary fibroblast cell line isolated from a patient with epidermoid carcinoma of the lung, HLF-A was grown in Eagle's minimum essential medium (EMEM) supplemented with 10% FBS.

Reagents

Unless otherwise specified, all reagents were purchased from Sigma (St Louis, Missouri, USA). Culture media, FBS and all additives were purchased from ATCC. Reagents for avidin/biotin immunoperoxidase staining were purchased from BioGenex (San Ramon, California, USA) and Vector Laboratories (Burlingame, California, USA).

Antibodies

β-Actin, a mouse monoclonal antibody (1:2500), was purchased from Sigma, and rabbit β-actin polyclonal antibody (1:2500) was purchased from Abcam (Cambridge, Massachusetts, USA). ERK1/2 kinases (p44/p42), a rabbit anti-rat polyclonal antibody (1:1000); phosphorylated ERK1/2 kinases (p44/p42 phosphorylated at residues Thr202/Tyr204), a rabbit polyclonal anti-human antibody (1:1000); and phosphorylated Akt kinase (Akt kinase phosphorylated at residues Ser 473), a rabbit polyclonal antibody (1:500) were purchased from Cell Signaling (Beverly, Massachusetts, USA). Akt kinase, a goat polyclonal antibody (1:500); cyclin E (HE12), a mouse monoclonal antibody (1:1000); cyclin D1, a rabbit polyclonal antibody (1:1000); TGF-α, a mouse monoclonal antibody (20 μg/ml); AR, a goat polyclonal antibody (20 μg/ml); and $p21^{Cip1}$ (p21) (C-19), a rabbit affinity purified polyclonal antibody (1:1000) were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). The fluorescently labeled secondary antibodies goat anti-rabbit and goat anti-mouse Alexa Flour 680 nm conjugates were purchased from Molecular Probes (Eugene, Oregon, USA), and goat anti-rabbit and goat anti-mouse IRDye 800 nm CW conjugates were purchased from Rockland (Gilbertsville, Pennsylvania, USA).

Coculture of pulmonary adenocarcinoma cells with CCD-19Lu or HLF-A fibroblasts

PAC cells were cocultured with fibroblasts in Transwell culture plates with inserts (Corning Transwell Inserts and Dishes, Corning, New York, USA) for Western blot analyses and in 24-well tissue culture plates with inserts (BD Falcon Cell Culture Inserts, Franklin Lakes, New Jersey, USA) for cell proliferation assays. This coculture system allows for the transfer of factors released into the cultured medium through the 0.4-μm insert pores in the membranes of the inserts, while preventing direct cell–cell contact between fibroblasts and cancer cells [7]. The PAC cells were seeded into 24-well plates (5×10^4 cells per well) or into 100-mm Transwell culture plates (3.5×10^6 per dish) in complete medium for 24 h (basal medium contains 10% FBS). Fibroblast cell lines were seeded in tissue culture inserts in complete DMEM (CCD-19Lu) or EMEM (HLF-A) media for 24 h. The fibroblasts and PAC cells were washed with phosphate-buffered saline and cocultured in DMEM/RPMI-1640 media (1:1, v/v) without FBS for cocultures of CCD-19Lu/NCI-H322 or DMEM/Ham's F-12 K modification media (1:1, v/v) without FBS for cocultures of CCD-19Lu/A549 for 5 days. The same conditions were applied for the HLF-A fibroblast cocultures with PAC cells, except that EMEM medium was used instead of DMEM medium. After 5 days of coculture, the fibroblasts on membrane culture insert were removed and proliferation of the epithelial cells was measured by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assay for the assessment of pulmonary adenocarcinoma cell proliferation

After 5 days of coculture, the fibroblasts on membrane culture inserts were removed and proliferation of the PAC cells was measured by MTT assay according to the

manufacturer's (Sigma-Aldrich) protocol. Briefly, the MTT test is based on the enzymatic reduction of the water-soluble tetrazolium salt MTT into water-insoluble formazan in live, metabolically active cells. The MTT (5 mg/ml) solution was added into wells with PAC cells. After 2 h of incubation, the medium was removed and the reaction product, a purple-colored formazan, was dissolved by 2-propanol. Absorbance was measured by a μQuant microplate spectrophotometer (MQX 200, Bio-Tek Instruments, Winooski, Vermont, USA). Data are mean values ± standard deviations (SD) of four replicates per group from three independent experiments expressed as normalized values relative to the control. One-way analysis of variance and Student's *t*-test were used to analyze the data ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Reverse transcription-polymerase chain reaction for the assessment of epidermal growth factor, transforming growth factor-α, amphiregulin and epidermal growth factor receptor mRNA

Total RNA was isolated by the Absolutely RNA Kit (Stratagene, La Jolla, California, USA). RNA samples were used to prepare cDNA as previously described [11]. The oligonucleotide primers employed for the polymerase chain reactions are shown in Table 1 [12,13]. Reactions were run on a PTC-200 thermal cycler (MJ Research, Watertown, Massachusetts, USA) with the following conditions: one cycle of 1 min at 96°C; 40 cycles × (94°C for 1 min; 58°C for 1 min; 72°C for 1 min); with the final extension for 10 min at 72°C. Polymerase chain reaction products were run on a 1.5% agarose gel for 2.15 h at 75 V. A 100-bp ladder (Life Technologies, Gaithersburg, Maryland, USA) was used as the marker. The gel was imaged by ethidium bromide staining using an Ultra Lum (Paramount, California, USA) TUI-5000 gel documentation system.

Determination of phosphorylated extracellular signal-regulated kinase 1/2, Akt kinases and cell cycle proteins expression in pulmonary adenocarcinoma cells after stimulation by fibroblast-conditioned medium

For the detection of phosphorylated ERK1/2 or Akt proteins in PAC cells, cells were seeded into 60-mm tissue culture dishes (1.2×10^6 cells) and incubated for 24 h in complete media. After overnight starvation in serum-free media, the PAC cells were treated with fibroblast-conditioned medium for 0.5, 3, 6 and 24 h. The conditioned media from fibroblasts were obtained from fibroblasts cultivated in 75-cm^2 tissue flasks with 70–80% density for 5 days in serum-free media (DMEM or EMEM). The media were centrifuged after collection and stored in the refrigerator at 4°C until used, but not longer than 3–4 days.

Western blot analysis for assessment of cell cyclin proteins in pulmonary adenocarcinoma cells

After coculture, the fibroblasts on inserts were removed and PAC cells were washed 2 times with ice-cold phosphate-buffered saline and lysed according to published procedures [14] in ice-cold Nonidet P-40 lysis buffer (20 mmol/l Tris pH 7.5, 200 mmol/l NaCl, 0.25% NP-40, 5 mmol/l ethylene diaminetetraacetic acid, 0.25% sodium deoxycholate, 1 mmol/l Na_3VO_4, 10 mmol/l NaF and 1 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml aprotinine) with brief sonication and centrifugation. Western blots were performed as described [14], except membranes were washed and incubated in diluted fluorescently labeled secondary antibody in blocking solution [Alexa Flour 680 nm goat anti-mouse or goat anti-rabbit 1:2500 (Molecular Probes); and IRDye 800 nm anti-mouse or anti-rabbit 1:5000 (Rockland)]. The signal was detected by scanning with an Odyssey infrared imaging system (LI-COR, Lincoln, Nebraska, USA).

Table 1 List of PCR primers

Primer	Sequence	Size (bp)	Source
EGF			
forward	5′-TATGTCTGCCGGTGCTCAGAA-3′	393	Maxim Biotech (San Francisco, California, USA)
reverse	5′-AGCGTGGCGCAGTTCCCACCA-3′		
EGFR			
forward	5′-CTCCGGTGCGTTCGGCAC-3′	351	Maxim Biotech
reverse	5′-GGTGCACCAAGCGACGGTC-3′		
AR			
forward	5′-TTGGACCTCAATGACACCTACTGTG-3′	478	Fernandes *et al.*[12]
reverse	5′-TGGACTTTTCCCCACACCGTTC-3′		
TGF-α			
forward	5′-CGCCCTGTTCGCTCTGGGTAT-3′	264	Schiemann *et al.*[13]
reverse	5′-AGGAGGTCCGCATGCTCACAG-3′		
Cyclophilin			
forward	5′-GCTGCCTGTGCACTCATGAA-3′	216	Ambion (Austin, Texas, USA)
reverse	5′-CAGTGCCATTGTGGTTTGTGA-3′		

EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TGF, transforming growth factor; AR, amphiregulin; PCR, polymerase chain reaction.

Densitometry analysis of protein expression from Western blots

Membranes were detected by scanning with an Odyssey infrared imaging system. Individual bands were selected, mean raw optical density were measured by Scion Image software (Scion Corporation, Frederick, Massachusetts, USA) and normalized to control. Data are representative of three independent Western blots and data from densitometry analysis are expressed as mean values ± SD of 10 readings per band and expressed as normalized values relative to the control.

Immunocytochemical analysis of the expression of cyclin D1, cyclin E and phosphorylated extracellular signal-regulated kinase 1/2 protein in pulmonary adenocarcinoma cells

Expression of cyclin E, cyclin D1 and phosphorylated ERK1/2 kinases in PAC cells was assessed immunocytochemically, according to published procedures [14]. Briefly, NCI-H322 and A549 cells were seeded onto four-well chamber slides (Nunc Lab-Tek II Chamber Slide System, Nalge Nunc International, Naperville, Illinois, USA) at 50% confluence in complete medium for 2 days. After 24 h starvation, the media was changed to (1) media without FBS, (2) media containing 10% FBS, (3) fibroblast-conditioned media from CCD-19Lu fibroblasts or (4) media without FBS with CCD-19Lu fibroblasts for 48 h. After fixation of the cells in acetone for 5 min, the primary antibody (cyclin D1 1:50, cyclin E 1:50 and phosphorylated ERK1/2 1:50) was added for 1 h, super sensitive link-biotinylated anti-rabbit or anti-mouse immunoglobulins (BioGenex) for 30 min and super sensitive label-peroxidase-conjugated streptavidin (BioGenex) for 30 min and incubated in a humid chamber at room temperature. Specific binding was visualized by staining with chromogen-diaminobenzidine (Vector Laboratories). The nuclei were lightly counterstained with Harris hematoxylin (Sigma). Nonspecific binding of the secondary antibody was examined by following the protocol described above, but without the addition of primary antibodies. No significant reaction occurred in the negative controls.

Results

Fibroblast-induced proliferation of pulmonary adenocarcinoma cells

To determine whether fibroblasts promote proliferation of PAC cells without cell–cell contact, we used the human PAC cell lines NCI-H322 and A549 with the human fibroblasts CCD-19Lu (from a healthy donor) and HLF-A (from a patient with epidermoid carcinoma of the lung) in an indirect coculture system as described in details in the Material and methods section. After 5 days in serum-free media, proliferation of PAC cells was analyzed by MTT assay. As Fig. 1 shows, increasing the number of CCD-19Lu fibroblasts enhanced proliferation of NCI-H322 and A549 cells. At densities of 2.5×10^4, 5×10^4 and 1×10^5 CCD-19Lu fibroblasts, proliferation of NCI-H322 cells was significantly increased by approximately 3.5-, 4.5- and 5.5-fold, respectively ($P < 0.001$). The proliferation of A549 cells was significantly induced when cocultured with higher amounts of CCD-19Lu fibroblasts (5×10^4 and 1×10^5 cells) by 1.5- and 2.5-fold, respectively ($P < 0.001$ and $P < 0.05$, respectively). Interestingly, HLF-A fibroblasts from a lung cancer patient were more potent than CCD-19Lu fibroblasts in stimulating PAC cells. At densities of 2.5×10^4, 5×10^4 and 1×10^5 HLF-A fibroblasts, the proliferation of PAC cells was significantly increased by 2-, 4- and 6.5-fold in NCI-H322, and 2-, 4- and 5.5-fold in A549, respectively ($P < 0.05$, $P < 0.01$, $P < 0.001$) as shown in Fig. 1. PAC cell proliferation was significantly inhibited by exposure of the cells to antibodies against TGF-α and AR (Fig. 1). At a density of 1×10^5 CCD-19Lu or HLF-A fibroblasts in the presence of antibodies against TGF-α (20 μg/ml) and AR (20 μg/ml), proliferation of PAC cells was significantly reduced by 0.5- and 0.3-fold in NCI-H322, and 0.8- and 0.3-fold in A549, respectively ($P < 0.001$). These data suggest that the observed proliferation of PAC cells was caused by the release of TGF-α and AR from the fibroblasts.

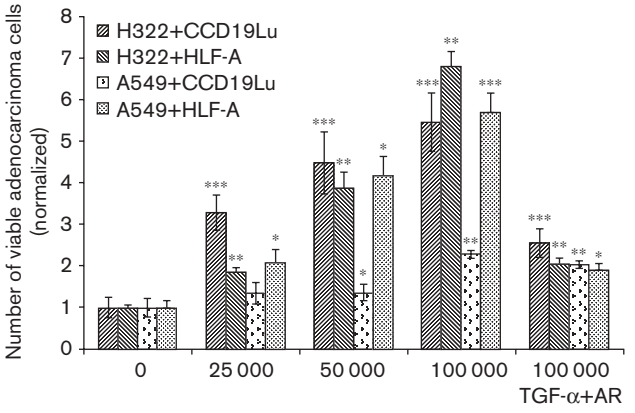
Analysis of mRNA expression for epidermal growth factor, amphiregulin, transforming growth factor-α and epidermal growth factor receptor in HLF-A, CCD-19Lu, NCI-H322 and A549 cell lines

All tested cell lines expressed mRNA for the epidermal growth factor receptor (EGFR) as well as for the growth factors AR and TGF-α (Fig. 2). The expression of mRNA for TGF-α in normal fibroblast cell line CCD-19Lu was higher than in the HLF-A fibroblast cell line isolated from a patient with epidermoid carcinoma, but lower as compared with each of the PAC cell lines. By contrast, mRNA for EGF was detected in NCI-H322 and A549 adenocarcinoma cells only, but not in fibroblast cell lines CCD-19Lu or HLF-A. Expression of mRNA for cyclophilin, a housekeeping gene, served as a positive and equal loading control. Negative controls without the M-MLV reverse transcriptase enzyme showed no detectable bands, confirming the absence of any contamination with genomic DNA. Our reverse transcription-polymerase chain reaction data suggest that release of the growth factors AR and TGF-α from the fibroblasts caused the observed paracrine stimulation of proliferation of PAC cells above base level proliferation sustained via autocrine stimulation by EGF, AR and TGF-α.

CCD-19Lu and HLF-A fibroblast-secreted factors stimulate proliferation of pulmonary adenocarcinoma cells via activation of mitogen-activated protein kinases and Akt

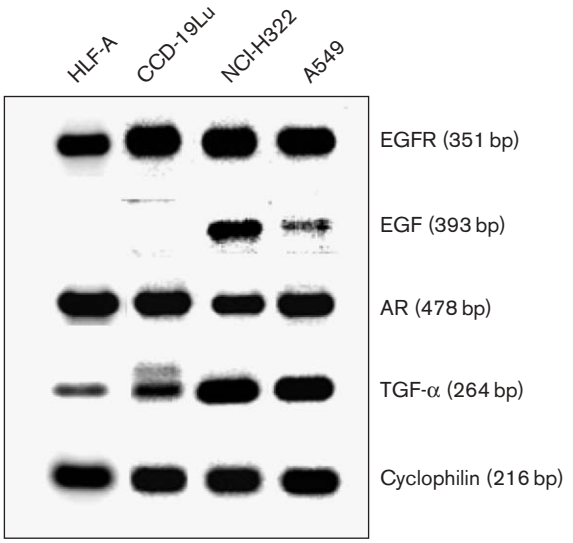
As ERK1/2 and Akt kinases play an important role in the regulation of cell growth, proliferation, apoptosis, differ-

Fig. 1



Results of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assay for the assessment of cell proliferation. The proliferation of NCI-H322 cells was significantly induced (3.5-, 4.5- and 5.5-fold, $P<0.001$) by CCD-19Lu fibroblasts, derived from a healthy human lung; however, higher effects (2-, 4- and 6.5-fold, $P<0.01$) were achieved when cocultured with HLF-A fibroblasts derived from the lung of an individual with epidermoid carcinoma. The proliferation of A549 cells was also significantly induced when cocultured with CCD-19Lu fibroblasts (1.5- and 2.5-fold, $P<0.05$ and $P<0.01$, respectively), but prominently increased by the presence of HLF-A fibroblasts (2-, 4- and 5.5-fold, $P<0.05$ and $P<0.001$, respectively). The proliferative response of both pulmonary adenocarcinoma cell lines to fibroblasts was significantly ($P<0.001$) reduced in the presence of antibodies against transforming growth factor (TGF)-α and amphiregulin (AR). Data are expressed as mean values ± standard deviations of four replicates of three independent experiments and expressed as normalized values relative to the control. *P*-values of the one-way analysis of variance and two-tailed Student's *t*-test were considered significant at $*P<0.05$, $**P<0.01$, $***P<0.001$.

Fig. 2



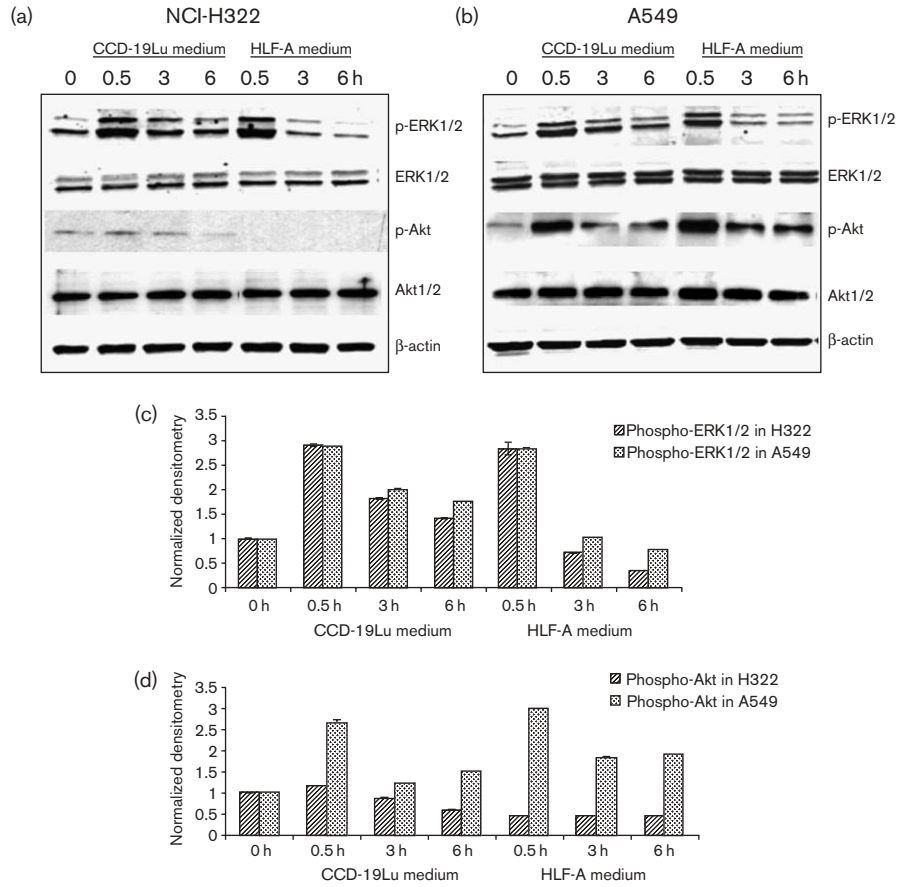
Reverse transcription-polymerase chain reaction for detection of the epidermal growth factor receptor (EGFR), and growth factors epidermal growth factor (EGF), amphiregulin (AR) and transforming growth factor (TGF)-α mRNA in HLF-A, CCD-19Lu, NCI-H322 and A549 cell lines. All tested cells expressed similar levels of mRNA for EGFR and AR. EGF mRNA was detected only in the pulmonary adenocarcinoma (PAC) cell lines NCI-H322 and A549. The relative levels of mRNA for TGF-α appeared higher in CCD-19Lu fibroblasts than in HLF-A fibroblasts, but lower as compared with the two PAC cell lines. Expression of mRNA for cyclophilin, a housekeeping gene, served as a positive and equal loading control for semiquantitative analysis.

entiation and responses to extracellular signals, we examined the activation of ERK1/2 and Akt kinases in PAC cells after culture in fibroblast-conditioned media. The PAC cells were seeded, starved in serum-free media for 24 h and treated with CCD-19Lu or HLF-A fibroblast-conditioned media for the indicated times (0.5, 3 and 6 h). Both PAC cell lines exposed to fibroblast-conditioned media from CCD-19Lu or HLF-A fibroblasts showed a transient increase in phosphorylation of ERK1/2 kinases (Fig. 3) with the highest phosphorylation levels at 0.5 h (3-fold each). Interestingly, A549 cell lines exposed to fibroblast-conditioned media from CCD-19Lu or HLF-A fibroblasts showed a temporary increase in phosphorylation of Akt kinase (Fig. 3b) with the highest phosphorylation levels at 0.5 h (2.5- and 3-fold, respectively). By contrast, phosphorylation of Akt kinase was not induced in NCI-H322 cells. These findings indicate that the proliferative response of both PAC cell lines observed in the MTT assays was mediated via activation of the ERK1/2 signaling cascade, whereas activation of Akt was an additional factor in the proliferative response of A549 cells.

Changes in the expression of cell cycle proteins in NCI-H322 and A549 cells cocultured with CCD-19Lu or HLF-A fibroblasts

To determine whether CCD-19Lu fibroblast cocultures modulate cell cycle regulation of PAC cells, we examined expression levels of the cell cycle proteins cyclin D1, cyclin E and cyclin-dependent kinase inhibitor (CDKI) p21 (Fig. 4). Expression levels of cyclin D1 protein in NCI-H322 and A549 cells were increased in the presence of both CCD-19Lu (1.5- and 3-fold, respectively) as well as HLF-A fibroblasts (1.5- and 3-fold, respectively) after 5 days of cocultures. In addition, the levels of cyclin E were also increased in NCI-H322 and A549 cells by the presence of pulmonary fibroblasts from a healthy individual, CCD-19Lu (3- and 1.5-fold, respectively) or a patient with epidermoid carcinoma, HLF-A (3.5- and 1.5-fold, respectively). The expression of cell cyclin proteins were increased, the expression levels of CDKI p21 protein was slightly increased in NCI-H322 cells treated with CCD-19Lu fibroblasts (2-fold), whereas no apparent change in p21 protein expression was detected in response to HLF-A fibroblasts.

Fig. 3



Results of Western blot analysis for the detection of phosphorylated and unphosphorylated extracellular signal-regulated kinase (ERK) 1/2 and Akt kinase proteins in pulmonary adenocarcinoma (PAC) cells. The PAC cells were exposed to fibroblast-conditioned media from CCD-19Lu or HLF-A fibroblasts for 0, 0.5, 3 and 6 h. Both cell lines showed increased activation of ERK1/2 kinases (3-fold each), with a maximum peak at 0.5 h (a and b). Activation of Akt kinase was detected only in A549 cells in response to fibroblast-conditioned media from CCD-19Lu or HLF-A cells (a and b), with maximum induction at 0.5 h (2.5- and 3-fold, respectively). The expression levels of total ERK1/2, Akt kinases and β-actin protein remained unchanged in both PAC cell lines, verifying equal loading. Data are representative of three independent Western blots. Data from densitometric analysis of the bands illustrated in the graphs are mean values ± standard deviations of 10 readings per band and are expressed as normalized values relative to the controls.

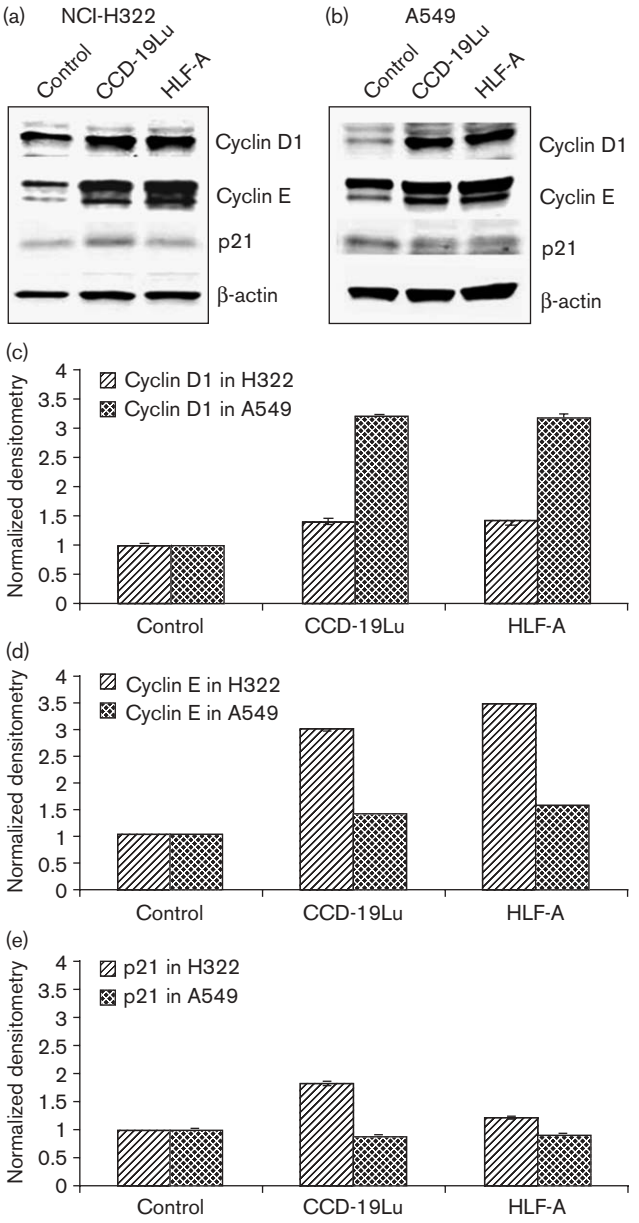
Changes in the expression of cell cycle proteins in NCI-H322 and A549 cells cultured with fibroblast-conditioned media

The short-term effects of fibroblasts on PAC cells proliferation were studied in NCI-H322 or A549 cells cultured in conditioned media from CCD-19Lu or HLF-A fibroblasts. PAC cells were harvested after 0.5, 3, 6 and 24 h treatments, and cyclin D1, cyclin E and CDKI p21 protein levels were assessed. As Fig. 5(a) shows, in NCI-H322 cells fibroblast-conditioned medium from CCD-19Lu fibroblasts induced expression of cyclin D1 at 3 and 6 h (1.5-fold, each), cyclin E at 6 and 24 h (1.5-fold, each), whereas the expression of p21 was not affected. NCI-H322 cells treated by conditioned medium from HLF-A fibroblasts showed an increase of cyclin D1 at 3 h (1.3-fold), cyclin E at 0.5–24 h (1.3-fold, each) and slight induction of p21 proteins levels at 24 h (1.3-fold) were observed (Fig. 5a). As Fig. 5(b) shows, conditioned medium from CCD-19Lu fibroblasts had only slight effects on the expression of cyclin D1 in A549 cells, cyclin E proteins and the expression of p21 protein was decreased at 6 and 24 h (0.5-fold, each). A549 cells treated by conditioned medium from HLF-A fibroblasts showed an increase of cyclin D1 and cyclin E protein expression at 0.5–6 h (1.3-fold, each), and slightly increased p21 protein expression at 3 h.

Immunocytochemical analysis of cyclin D1, cyclin E and phosphorylated extracellular signal-regulated kinase 1/2 protein expression in pulmonary adenocarcinoma cells treated by CCD-19Lu fibroblasts or fibroblast-conditioned media

To detect expression of cyclin D1, E and phosphorylated ERK1/2 kinase proteins, we performed immunocyto-

Fig. 4



Results of Western blot analysis for the detection of cyclin D1, cyclin E and p21 protein expression in pulmonary adenocarcinoma (PAC) cells. Cocultures with each fibroblast cell line for 5 days increased expression levels of cyclin D1 and cyclin E in NCI-H322 cells (1.5- and 3.5-fold, respectively) and A549 cells (3- and 1.5-fold, respectively). CCD-19Lu fibroblasts elevated the expression level of cyclin-dependent kinases inhibitor, p21 (2-fold) only in NCI-H322 cells while no significant change in p21 protein level was detected by HLF-A fibroblasts. β-Actin proteins were not changed in PAC cells, verifying equal loading. Densitometry analysis of cyclin D1 (c), cyclin E (d) and p21 (e) protein expression levels normalized to the control. Data are representative of three independent Western blots, and data from densitometry analysis are expressed as mean values ± standard deviations of 10 readings per band and expressed as normalized values relative to the control.

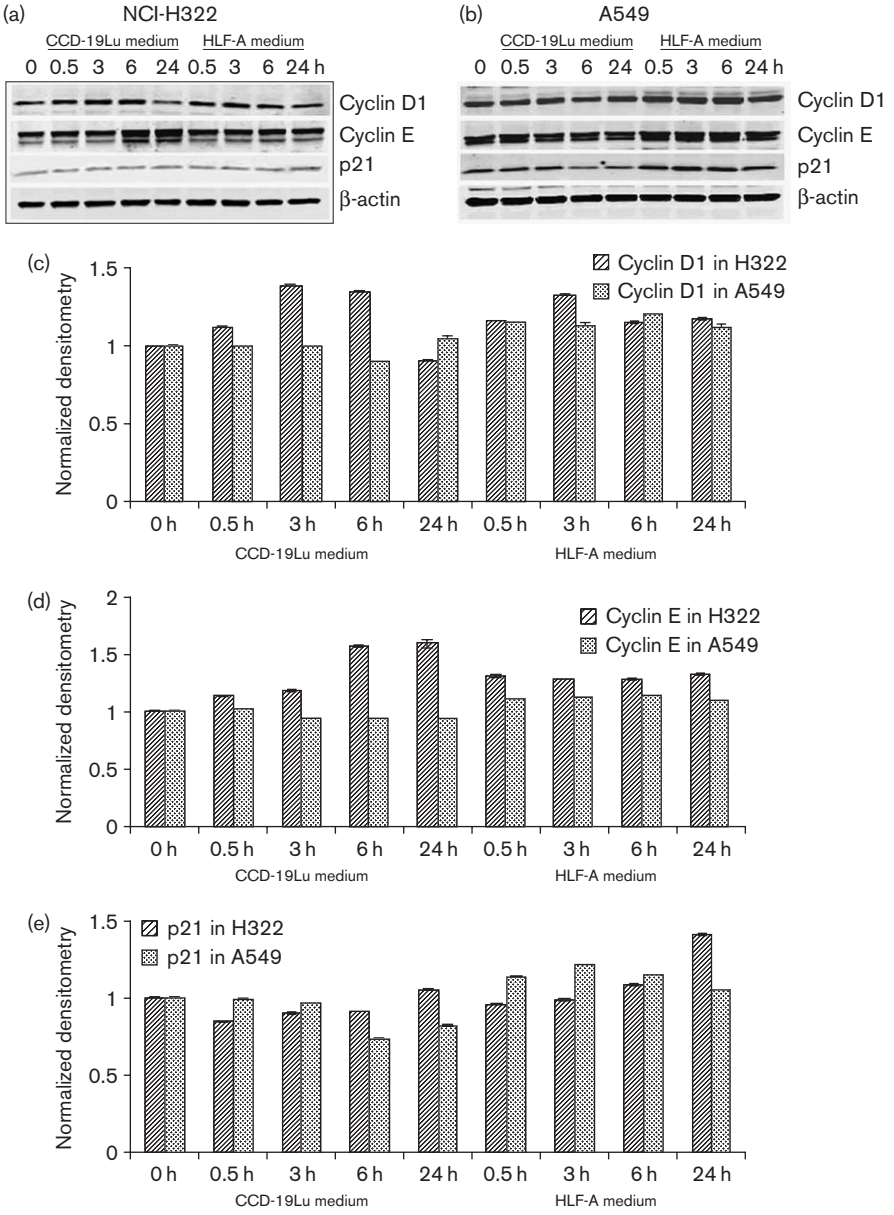
chemistry on NCI-H322 and A549 cells using antibodies specific for cyclin D1, cyclin E and phosphorylated ERK1/2. The PAC cells were treated with either serum-free medium (Fig. 6a–c), fibroblast-conditioned media from CCD-19Lu fibroblasts (Fig. 6d–f) or in direct coculture with CCD-19Lu fibroblast (arrows, Fig. 6g–i) or in 10% FBS as a positive control for the expression of cell cycle proteins and phosphorylated ERK1/2 (data not shown). Positive immunoreactivity for cyclin D1 (Fig. 6d and g) and cyclin E (Fig. 6e and h) was observed in the nuclei and cytoplasm (brown color) in NCI-H322 cells treated with fibroblast-conditioned media as well as in direct coculture with fibroblasts. Positive immunoreactivity for phosphorylated ERK1/2 kinase proteins was also detected (Fig. 6f and i). Either no signal or a very weak signal was detected in the cytoplasm or nuclei for cyclin D1 and cyclin E (Fig. 6a and b), or in the cytoplasm for phosphorylated ERK1/2 (Fig. 6c) in serum-deprived cells not cocultured with fibroblasts. To verify the specificity of the observed immunoreactions, negative control slides not exposed to the primary antibodies were used.

Discussion

To study the effects of fibroblasts on the tested PAC cells, we used an indirect coculture system without cell–cell contact. The membrane between the cells represented a physical barrier and the interactions were achieved by diffusion of growth factors through the membrane. Using this coculture system of fibroblasts and PAC cells, we demonstrated that: (1) CCD-19Lu fibroblasts stimulated the growth of NCI-H322 and A549 cells, (2) increased numbers of CCD-19Lu fibroblasts enhanced proliferation of PAC cells, (3) HLF-A fibroblasts had stronger stimulating effects on the proliferation of PAC cells, (4) proliferation of PAC cells in response to coculture with fibroblasts was inhibited by antibodies against TGF-α and AR, (5) expression levels of cell cycle proteins cyclin D1, cyclin E and CDKI p21 in the PAC cells correlated with their proliferation responses, (6) the fibroblast-induced proliferation of both PAC cell lines involved activation of ERK1/2 kinases and (7) additional activation of phosphorylated Akt kinase in A549 cells only.

The presence of mRNA for EGF in both PAC cell lines, but not in the fibroblasts, suggests that growth factors other than EGF were responsible for the observed stimulation of PAC cells by fibroblasts. Both TGF-α and AR have documented growth-stimulating effects on non-small-cell lung cancer cells, including PACs [12,15,16]. While it has been documented that these growth factors as well as EGF are produced by the cancer cells and normal lung epithelial cells to stimulate their growth in an autocrine manner, our data suggest an additional paracrine stimulation of PAC cells via TGF-α and AR released from pulmonary fibroblasts. This interpretation is supported by the observed inhibition of fibroblast-induced PAC cell proliferation by antibodies against TGF-α and AR. The participation of these two growth

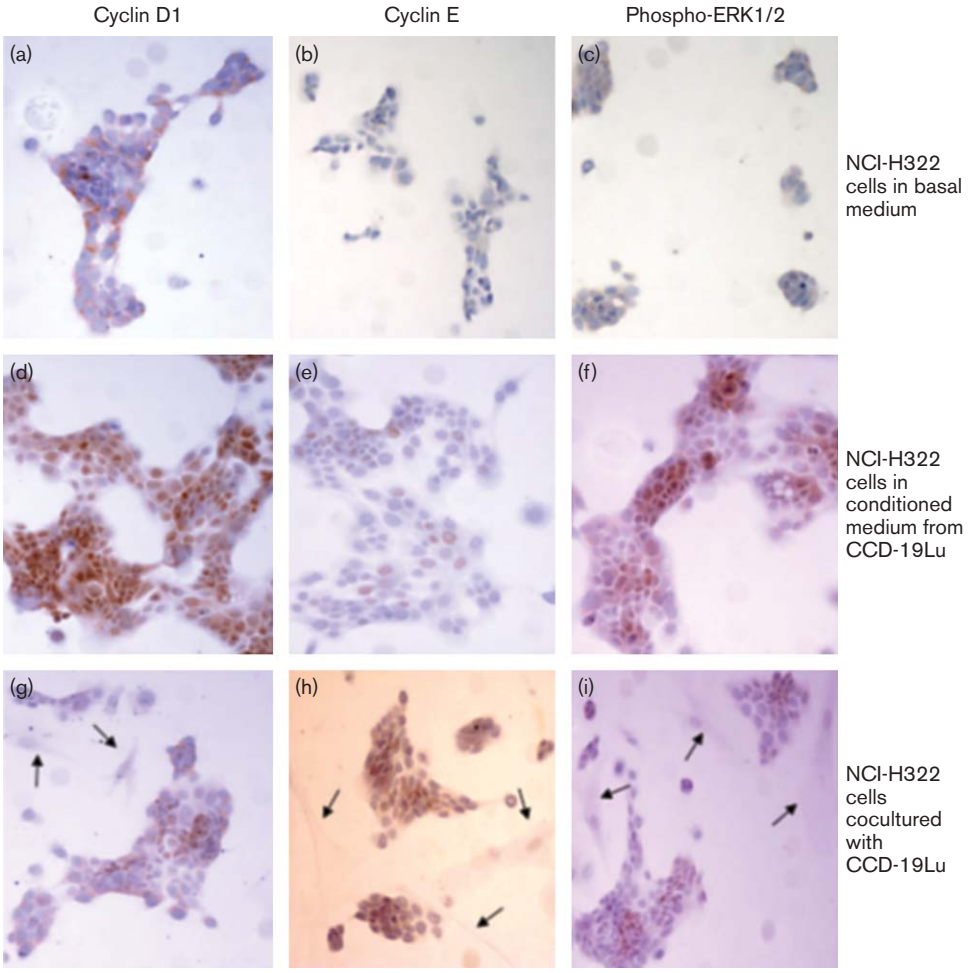
Fig. 5



Results of Western blot analysis for the detection of cyclin D1, cyclin E and p21 protein expression in pulmonary adenocarcinoma (PAC) cells after short-term culture with fibroblast-conditioned media. PAC cells were harvested after 0.5, 3, 6 and 24 h of exposure to fibroblast-conditioned media, and cyclin D1, cyclin E and cyclin-dependent kinases inhibitor p21 protein levels were assessed. (a) In NCI-H322 cells, fibroblast-conditioned medium from CCD-19Lu fibroblasts induced expression of cyclin D1 at 3 and 6 h, and cyclin E at 6 and 24 h, whereas the expression of p21 was not affected. NCI-H322 cells treated by conditioned medium from HLF-A fibroblasts showed an increase of cyclin D1 at 3 h, cyclin E at 0.5–24 h and a slight induction of p21 proteins levels at 24 h. (b) In A549 cells, treatment of fibroblast-conditioned medium from CCD-19Lu fibroblasts had only slightly induced the expression of cyclin D1 and cyclin E, whereas the expression of p21 was decreased at 6 and 24 h. A549 cells treated by conditioned medium from HLF-A fibroblasts showed an increase of cyclin D1 and cyclin E protein expression at 0.5–6 h, and a slight increase in p21 protein expression at 3 h. β-Actin proteins were not changed in PAC cells, verifying equal loading. Densitometric analysis of the bands for cyclin D1, cyclin E and p21 protein expression levels is illustrated in the graphs (c–e). Data are representative for three independent Western blots. Densitometry values in the graphs are mean values ± standard deviations of 10 readings per band and are expressed as normalized values relative to the controls.

factors in this paracrine loop is particularly interesting in light of a recent report that increased serum levels of TGF-α and AR in lung cancer patients is predictive of their poor response to the tyrosine kinase inhibitor gefitinib [16]. Small-molecule tyrosine kinase inhibitors, such as gefitinib, were initially hailed as miracle drugs for

Fig. 6

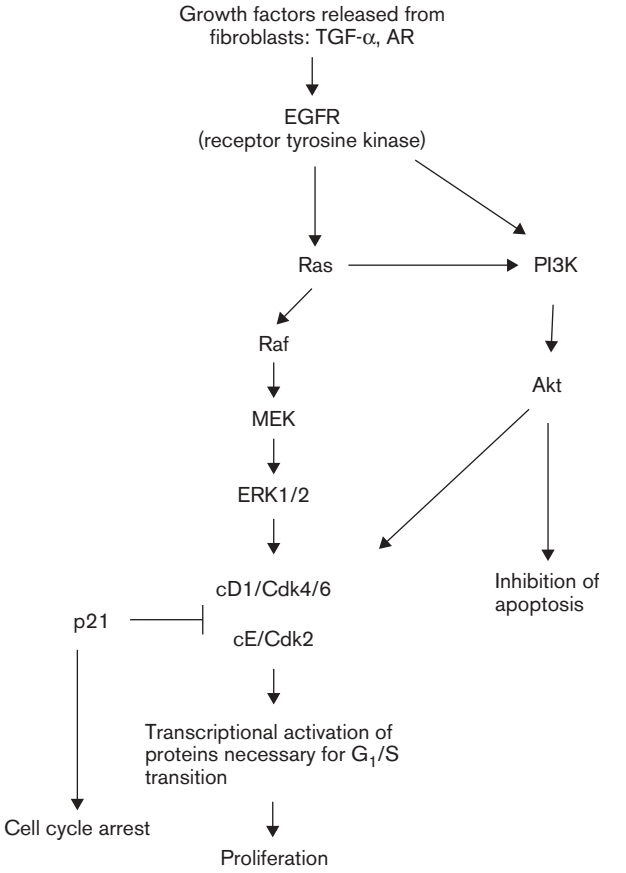


Representative results of immunocytochemical analysis for the detection of cyclin D1, cyclin E and phosphorylated extracellular signal-regulated kinase (ERK) 1/2 kinases in NCI-H322 cells after treatment with CCD-19Lu fibroblast and fibroblast-conditioned medium. Cells were seeded in four-well chamber slides and treated with either serum-free media (a–c), fibroblast-conditioned media from CCD-19Lu fibroblasts (d–f) or in direct coculture with CCD-19Lu fibroblasts (arrows) (g–i). Immunoreactivity was visualized using an avidin–biotin peroxidase stain. Increased expression of cyclin D1 (d and g), cyclin E (e and h) and phosphorylated ERK1/2 kinases (f and i) was seen in the nuclei (brown color) of adenocarcinoma NCI-H322 cells, when cocultured with fibroblasts as well when cocultured with fibroblast-conditioned media. Increased levels of tested proteins confirmed previous data of increased proliferation of pulmonary adenocarcinoma (PAC) cells stimulated by fibroblasts. The cells were counterstained with hematoxylin (blue color), $\times 160$ magnification. The data are representative for two independent experiments. Similar positive immunoreactivity was observed in A549 PAC cells cultured in the presence of CCD-19Lu and HLF-A fibroblast cell lines as well from both fibroblast-conditioned media (data not shown).

the treatment of non-small-cell lung cancer [17,18], following reports that they blocked the proliferation of lung cancer cell lines dependent on the EGFR/ERK1/2/Akt pathway [19,20]. These agents, however, disappointed in clinical trials with only a small subset of PAC patients who typically were non-smokers showing positive responses. AR has been shown to inhibit apoptosis in human PAC cell lines through an insulin-like growth factor-1 receptor-dependent pathway and independent of binding to the EGFR [21]. Accordingly, the paracrine stimulation of PAC cells via AR released from pulmonary fibroblasts observed by us may contribute to the reported resistance to gefitinib of patients with high serum levels of this growth factor [16].

The expression of the cell cycle proteins cyclin D1 and E correlated with the proliferation of lung PAC cells after 5 days in indirect cocultures, whereas protein levels for the inhibitor of cell cycle progression, p21, remained unchanged. The expression level of cyclin D1 and E proteins were indicative of G_1/S transition [22–24] in NCI-H322 and A549 cells. Short-term exposure of PAC cells to fibroblast-conditioned media correlated with the MTT results, showing higher stimulation of NCI-H322

Fig. 7



Simplified proposed signaling pathways involved in the proliferative response of pulmonary adenocarcinoma cells to the growth factors transforming growth factor (TGF)-α and amphiregulin (AR) released from lung fibroblasts. EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol 3′-kinase; MEK, ERK kinase; ERK, extracellular signal-regulated kinase; Cdk, cyclin-dependent kinase.

cells by CCD-19Lu fibroblasts by higher expression of cyclin D1 and cyclin E levels. By contrast, A549 cells showed higher proliferation after stimulation by HLF-A fibroblasts, which correlated with the higher expression levels of cyclin D1 and cyclin E after short-term exposure to fibroblast-conditioned media. The different responsiveness of these two PAC cell lines may be caused by different expression levels of receptors and autocrine growth factors in these phenotypically different PAC cell lines [15].

Our data show that release of the growth factors AR and TGF-α from fibroblasts activated ERK1/2 kinases followed by activation of cell cycle proteins in both PAC cell lines, thus leading to their increased proliferation. In addition to ERK1/2, the activation of tyrosine kinase receptors, such as the EGFR, by growth factors may activate phosphatidylinositol 3-kinases that in turn stimulate the serine/threonine kinase Akt (Fig. 7). This pathway may control cell survival, inhibition of apoptosis and cell proliferation. Activity of Akt kinase was increased in A549 cells in response to fibroblast-conditioned media, with HLF-A fibroblasts having a more pronounced effect than CCD-19Lu. By contrast, Akt was not activated by fibroblast-conditioned media in NCI-H322 cells. The activation of the Akt kinase in addition to ERK1/2 in A549 correlated with the higher responsiveness of these cells to HLF-A fibroblasts. Activation of Akt has also been reported in response to nicotine or its carcinogenic derivative 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in human bronchial and bronchiolar epithelial cells [25].

The observed higher potency to stimulate PAC cell growth of fibroblasts from a donor with lung cancer is in accordance with reports that cancer cells enhance the release of growth factors and angiogenic factors from fibroblasts [26–28]. While these publications on interactions between tumor cells and surrounding stromal elements have focused on changes in the fibroblasts caused by the cancer cells, our current data are the first to address a direct stimulation of PAC cell growth caused by growth factors released from surrounding fibroblasts. Such paracrine loops need to be considered when designing test systems for the identification of effective anticancer agents.

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