Inhibition of Apoptosis by Amphiregulin via an Insulin-like Growth Factor-1 Receptor-dependent Pathway in Non-small Cell Lung Cancer Cell Lines*  

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Several abnormalities in the insulin-like growth factor-1 (IGF1) and erbB receptors pathways stimulate the growth and survival of lung cancer cells, but their mechanisms of action and cooperation are poorly understood. In this report, we have identified a new mechanism of apoptosis inhibition by amphiregulin through an IGF1-dependent survival pathway in non-small cell lung cancer (NSCLC) cells: amphiregulin activates the IGF1 receptor that in turn induces the secretion of amphiregulin and IGF1. In the absence of serum, the NSCLC cell line H358 resists apoptosis and secretes factors protecting the NSCLC cell line H322 from serum deprivation apoptosis. IGF1 receptor inhibitor AG1024 as well as epidermal growth factor receptor inhibitors AG556 and ZD1839 restore apoptosis in H322 cells cultured in H358-conditioned medium. Accordingly, the anti-apoptotic activity of H358-conditioned medium is completely abolished after incubation with anti-amphiregulin neutralizing antibody and only partially with anti-IGF1 neutralizing antibody. H358-conditioned medium and amphiregulin induce IGF1 receptor phosphorylation in H322 cells, which is prevented by anti-amphiregulin neutralizing antibody but not by AG556 or ZD1839. H358 cells secrete a high level of amphiregulin that, in combination with IGF1, prevents serum deprivation apoptosis. Finally, IGF1 receptor inhibitor blocks amphiregulin and IGF1 release by H358 cells.

In lung cancer, well characterized molecular abnormalities affect cell cycle control and apoptosis (1); in addition, a variety of peptide hormones and growth factors induce autocrine or paracrine stimulation of cell survival, proliferation, migration, and angiogenesis. Although tumor growth of small cell lung cancer is mainly mediated by neupeptides such as bombesin-related peptides that bind to G protein-coupled receptor, the major autocrine/paracrine loops for non-small cell lung cancer (NSCLC) cells involve growth factors that bind to tyrosine kinase receptors (2).

The erbB receptor family contains four distinct members: the epidermal growth factor receptor (EGFR, erbB-1/HER1), erbB-2/neu (HER2), erbB-3 (HER3), and erbB-4 (HER4). These receptors consist of an extracellular domain containing the ligand-binding domain, a hydrophobic transmembrane domain, and an intracellular domain conferring tyrosine kinase activity (3). Binding of specific ligands induces homo- or heterodimerization of the receptors within members of the erbB family (4) and activates distinct intracellular signal transduction pathways and biological responses, including cellular proliferation, differentiation, migration, and survival. Five ligands of EGFR, the epidermal growth factor (EGF), transforming growth factor α (TGFα), amphiregulin (AR), heparin-binding EGF (HB-EGF) and betacellulin, can be produced by tumorigenic and, to a lesser extent, by non-tumorigenic human lung epithelial cells (5–7). As most cells of epithelial origin, NSCLC cells overexpress members of the erbB receptor family; in particular the EGFR and erbB-2/neu, but the prognostic impact of this over-expression is still controversial (5, 6). Overexpression of TGFα and AR has been associated with shortened survival of patients with NSCLC (5, 6, 8, 9).

Type 1 insulin like-growth factor receptor (IGF1-R) is also expressed in human lung tumors (10, 11). The IGF1-R is composed of two extracellular α subunits with the ligand-binding domain and two β subunits that contain a single transmembrane domain and an intracellular domain with the tyrosine kinase activity. IGF1-R binds insulin-like growth factor-1 (IGF1) and IGF2. High levels of circulating IGF1 detected in patients with lung cancers (12, 13) suggested that the IGF1/IGF1-R pathway could be involved in the pathogenesis of this disease. IGF1 is a potent stimulator of mitogenesis (14, 15) and can promote cellular differentiation and transformation (16–18). Furthermore, IGF1 is a major survival factor and protects cells from apoptosis under a wide variety of circumstances (19, 20), including cytotoxic agents (21–25), overexpression of myc (26), caspase activation (27), irradiation with UV-B (28), brain ischemia (29), and serum withdrawal (27, 30–33).

Cross-talk between the IGF1-R, the EGFR, and their ligands has been reported. Stimulation of IGF1-R signaling interferes with the antitumoral activity of EGFR inhibitor (34) or of Trastuzumab, a monoclonal antibody used clinically to inhibit the growth of erbB-2/neu-expressing tumors (35). In addition, prolonged activation of the intracellular kinase ERK2 by EGF binding epidermal growth factor; IGF1, insulin-like growth factor-1; IGF1-R, insulin-like growth factor-1 receptor; IGF2, insulin-like growth factor-2; PDGFR, platelet-derived growth factor receptor; TGFα, transforming growth factor α; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay.

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EXPERIMENTAL PROCEDURES

Cell Culture, Serum Withdrawal, and H358-conditioned Medium—The human H358, H322, A549, HEL, K562, and Jurkat cell lines were cultured in RPMI 1640 medium; H69, H1417, and H2170 cells in RPMI medium supplemented with glucose (2.5 g/liter), HEPES (10 mM), sodium pyruvate (1 mM), CALU-1 and SAOS cells in McCoy’s medium; MCF7 and HeLa cells in Dulbecco’s modified Eagle’s medium; H810, H720, and H2106 cells in Hite’s medium; and NBHE cells in SAGM medium. Media were supplemented with 10% fetal calf serum, and cells were cultured at 37 °C in a humidified atmosphere of 5% CO2. All products were purchased from Invitrogen (Cergy Pontoise, France).

For serum deprivation, cells were rinsed once in PBS and cultured for at least 8 h in serum-free medium, which was then changed, and the culture continued in serum-free medium or in serum-free conditioned medium for the desired time period. Conditioned media (CM) from cells were collected at 72 h after serum-free culture, centrifuged (5000 g for the desired time) and stored at −80 °C until use.

Anti-human EGF or AR mouse monoclonal, anti-human TGF β or IGF1 goat polyclonal (Oncogene Research Products, Fontenay sous Bois, France) neutralizing antibodies were incubated overnight at 4 °C in H358 CM. The immunonutralized H358 CM was then added to H322 cells for 96 h.

Quantification of Apoptotic Cell Death—The morphological changes related to apoptosis were assessed by fluorescence microscopy after Hoechst 33342 (5 µg/ml) staining of cells (Sigma-Aldrich), and the percentage of apoptotic cells was scored after counting at least 200 cells.

In some experiments, active caspase-3 was detected by flow cytometry using phycoerythrin-conjugated monoclonal active caspase-3 antibody kit (BD Pharmingen) following the manufacturer’s instructions. Analysis was performed on a Becton Dickinson FACScan flow cytometer. Red fluorescence (phycoerythrin, FL-2) was detected at 575 nm.

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Fig. 1. Effect of serum withdrawal on apoptosis in H358 and H322 NSCLC cells. Serum deprivation induces apoptosis in H322 but not in H358 NSCLC lines. A, kinetics of apoptosis induced by serum withdrawal in H322 (open circles) or H358 cells (filled circles). Percentage of apoptosis was assessed by fluorescence microscopy after Hoechst staining of the cells. Results are expressed as the mean ± S.D. of three independent experiments. B, agarose gel electrophoresis of DNA extracted from H322 or H358 cells cultured in RPMI-serum 10% (0) in serum-free medium (0). DNA from H358 cells treated for 24 h with 100 nM staurosporine was used as positive control of apoptotic DNA fragmentation (+). C, Western blot analysis of the p12 fragment of active caspase-3 in H322 or H358 cells cultured in RPMI-serum 10% (0) or serum-free medium (0) or in serum-free conditioned medium from H358 cells (CM). Percentages of apoptosis are expressed as the mean ± S.D. of three independent experiments. ***, statistically more significant than H358 CM; p < 0.01.
Fig. 2. Effect of tyrosine kinase receptors inhibition on serum deprivation apoptosis in H322 cells. H322 cells were cultured in RPMI medium supplemented with 10% serum (10), serum-free medium (0), or conditioned medium from H358 cells (CM). The activity of indicated concentrations of AG825 (A), AG1295 (B), AG1024 (C), AG556 (D), and ZD1839 (E) was normalized at 100. Apoptosis was analyzed by fluorescence microscopy after Hoechst staining of cells and by detection of the p12 active fragment of caspase-3 by immunoblotting. Percentage of apoptosis detected in serum-free medium condition (0) was normalized at 100. Apoptosis was expressed as a rate of apoptosis in serum-free medium and as the mean ± S.D. of three independent experiments.

Table I

Effect of tyrosine kinase receptors inhibition on serum deprivation apoptosis in H358 cells.

The activity of tyrphostins AG556, AG1024, AG825, or AG1295 on apoptosis in H358 cells cultured in medium supplemented (10% serum) or not (0% serum) with serum was analyzed. Apoptosis was detected by fluorescence microscopy after Hoechst staining of cells. The percentage of apoptosis was expressed as the mean ± S.D. of at least three independent experiments.

| Receptor | Inhibitor | Concentration | 10% serum | 0% serum |
|----------|-----------|---------------|-----------|----------|
| EGFR     | Control   | 0.9 ± 0.1     | 1.9 ± 1.3 |
|          | AG 556    | 2             | 4.1 ± 0.0 |
|          | AG 1024   | 2.2 ± 1.3     | 4.65 ± 14.0 |
|          | AG 825    | 1.7 ± 1.2     | 29.3 ± 0.2 |
|          | AG 1295   | 2.3 ± 1.2     | 4.4 ± 0.6 |
| PDGFR    | AG 1295   | 4.0 ± 0.8     | 4.7 ± 1.2 |

Fig. 3. Effect of tyrosine kinase receptor inhibition on serum deprivation apoptosis in H358 cells. Western blotting analysis of pro-caspase-3 proteolytic activation in its p12 active fragment in H358 cells cultured in serum-free medium in the presence of indicated concentrations in μM of tyrphostins AG556, AG825, AG1024, or AG1295.

Table II

Effect of neutralization of the members of the EGF and IGF families on the anti-apoptotic activity of H358-conditioned medium.

H358-conditioned medium (CM) was preincubated with neutralizing antibodies, before being added to serum-deprived H322 cells for 96 h. Apoptosis was detected by fluorescence microscopy after Hoechst staining of cells. The percentage of apoptosis was expressed as a rate of serum deprivation apoptosis in H322 cells. Data represent the mean ± S.D. of at least three independent experiments.

| ND50 | Used concentrations | Apoptosis |
|------|---------------------|-----------|
|      | μg/ml               | %/control |
| 0    | 0                   | 100       |
| CM   | 7.1 ± 8.8           |           |
| CM + Ig | 3.4 ± 6.6        |           |
| CM + anti-EGF Ab | 4.4 ± 6.2 |           |
| Anti-TGFα Ab | 11.3 ± 10.8 |           |
| Anti-AR Ab | 45.9 ± 8.6 |           |
| CRM197 (HB-EGF) | 104.2 ± 16.7 |           |
| Anti-BTC Ab | 16.9 ± 15.0 |           |
| Anti-IGF1 Ab | 22.1 ± 19.7 |           |
| Anti-IGFII Ab | 55.1 ± 19.3 |           |

H358 NSCLC Cells Are Resistant to Serum Deprivation Apoptosis and Secrete Anti-apoptotic Factors—After 96 h of culture in serum-free medium, H322 cells underwent apoptosis as re-
AR and IGF1 concentrations were measured in H358, H322, or other cell line-conditioned media using ELISA detection. Five million cells were incubated during 72 h in serum-free medium. Data are expressed as the mean ± S.D. of at least three independent experiments.

| Cell lines       | AR (pg/ml) | IGF1 (pg/ml) |
|------------------|------------|--------------|
| H358 NSCLC       | 5803 ± 1635| 920 ± 113    |
| H322 NSCLC       | 100 ± 11   | 810 ± 14     |
| CALU-1 NSCLC     | 118 ± 17   | 810 ± 127    |
| A549 NSCLC       | 129 ± 13   | 487 ± 217    |
| H2170 NSCLC      | 105 ± 18   | 690          |
| H69 SCLC         | 133 ± 7    | 70 ± 14      |
| H1417 SCLC       | 152 ± 10   | 49 ± 2       |
| H810 LCNEC       | 195 ± 60   | 150          |
| H2106 LCNEC      | 813 ± 46   | 60           |
| H720 Human lung atypical carcinoid | 145 ± 25 | 245 ± 78 |
| NHBE Normal human bronchial epithelial cells | 85 ± 12 | 775 ± 78 |
| SAOS Human osteosarcoma | 178 ± 8 | 745 ± 149 |
| MCF7 Human breast adenocarcinoma | 100 ± 12 | 440 ± 170 |
| HEla Human cervix adenocarcinoma | 82 ± 20 | 365 ± 21 |
| HEL Human erythroleukemia | 124 ± 18 | 215 ± 92 |
| K562 Human chronic myelogenous leukemia | 175 ± 118 | 805 ± 35 |
| JURKAT Human T cell leukemia | 10 365 | 35 |
| CHO Chinese hamster ovary | 11 50 | 40 |

* SCLC, small cell lung cancer; LCNEC, large cell neuroendocrine lung cells.

** Fig. 4. AR and IGF1 recombinant proteins inhibit serum-deprived apoptosis in H322 cells. H322 cells were cultured in medium supplemented with 10% serum (10), in serum-free medium (0), in conditioned medium from H358 cells (CM), or in serum-free medium supplemented with the indicated concentrations of AR or IGF1 recombinant proteins. Apoptosis was analyzed by immunolabeling of active caspase-3 and flow cytometry detection. Percentage of apoptosis was expressed as a rate of apoptosis detected in serum-free medium (0) or in conditioned medium from H358 cells (CM), or in a medium supplemented with 10% serum, in serum-free medium or in H358 CM, normalized at 100. Data represent the mean ± S.D. of at least three independent experiments. *, p < 0.05; **, p < 0.005; statistically less significant than serum-free medium condition (0).

Seleved by morphological change (Fig. 1A). These changes coincided with DNA fragmentation visualized after agarose gel electrophoresis (Fig. 1B) and proteolytic cleavage of procaspase-3 in its p12 active fragment (Fig. 1C). Conversely, no evidence of cell death was detected in H358 cells, even after 120 h of serum deprivation (Fig. 1, A–C).

To determine whether the resistance of H358 cells was due to the constitutive activation of a survival pathway or whether those cells secrete anti-apoptotic factors, serum-free conditioned medium from H358 cells (H358 CM) was collected and used for culturing H322 cells. H358 CM inhibited apoptosis of H322 cells, evaluated by the absence of morphological changes or cleavage of caspase-3, after 96 h of culture (Fig. 1D). This effect was dose-dependent (data not shown).

** Inhibition of Serum Deprivation Apoptosis in H322 and H358 NSCLC Cell Lines by EGF and IGF1 Receptors—Human lung cancer cells are known to express EGFR, erbB-2/neu, IGF1-R, and platelet-derived growth factor receptors (PDGFRs). We first considered the possible involvement of these receptors in the survival of H322 cells cultured in H358 CM. Serum-deprived H322 cells were incubated in medium supplemented with 10% serum, in serum-free medium or in H358 CM in the presence of several highly specific inhibitors (Fig. 2). Tyrophostin AG825 and AG1295, which inhibited respectively the erbB-2/neu and the PDGFR, did not modify the survival of H322 cells cultured in the presence of serum, in serum-free medium, or in H358 CM (Fig. 2, A and B). The IGF1-R inhibitor tyrphostin AG1024 (1 μM) did not modify apoptosis in H322 cells cultured in the presence or in the absence of serum, but restored, in a dose-dependent manner, apoptosis when cells are cultured in H358 CM (Fig. 2C). Both EGFR inhibitors tyrophostin AG556 (20 μM) and quinazolin ZD1839 (500 nM) did not modify apoptosis of the cells in the presence of serum, increased the level of apoptosis of the cells when cultured in serum-free medium, and restored apoptosis in H322 cells cultured in H358 CM (Fig. 2, D and E).

We then analyzed the survival of H358 cells cultured in serum-free medium in the presence or absence of the same inhibitors (Table I). In the presence of serum none of the inhibitors induced apoptosis of H358 cells. In contrast, the EGFR inhibitor tyrphostin AG556 (10 μM) as well as the IGF1-R inhibitor tyrophostin AG1024 (10 μM) restored H322 cells apoptosis when cultured in serum-free medium (Table I). Conversely, concentration of up to 20 or 40 μM of the erbB-2/neu inhibitor tyrophostin AG825 and the PDGFR inhibitor tyrophostin AG1295 had no effect on H358 cell survival (Table I).
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A

H322

IP: IgG

| Time (min) | WB: phospho-Tyr |
|-----------|----------------|
| 5         | -              |
| 15        | -              |
| 30        | -              |

WB: IGF1-R β subunit

H322 CM + IGF1 AR

B

H322

IP: IgG

WB: phospho-Tyr

WB: IGF1-R β subunit

C

H358

IP: IgG

WB: phospho-Tyr

WB: IGF1-R β subunit

CM + IGF1 AR

Fig. 5. Effect of H358-conditioned medium, AR, and IGF1 on IGF1-R tyrosine phosphorylation. Serum-deprived H322 cells were incubated: A, for 30 min in serum-free medium (−), in serum-free medium with IGF1 50 ng/ml (IGF1), or for indicated times in serum-free H358 CM (CM); B, for 30 min in serum-free medium (−), in H358 CM (CM), in H358 CM preincubated with anti-AR neutralizing antibody (CM + Ac aAR), or in serum-free medium with 50 ng/ml of IGF1 (IGF1) or AR (AR), prior to detergent lysis and immunoprecipitation of the IGF1-R β subunit. C, serum-deprived H358 cells were incubated for 30 min in serum-free medium (−), in H358 CM (CM), or in serum-free medium with 50 ng/ml of IGF1 (IGF1) or AR (AR), prior to detergent lysis and immunoprecipitation of the IGF1-R β subunit. Tyrosine phosphorylation was visualized by protein immunoblotting with monoclonal anti-phosphotyrosine IgG as described (upper lane). Equal loading of immunoprecipitated proteins was confirmed by reprobing immunoblots with polyclonal anti-IGF1-R β subunit antibody (lower lane). IgG, immunoglobulin control for immunoprecipitation. Data are representative of at least three separate experiments.

The role of autocrine/paracrine factors in the context of survival signaling has been shown for several growth factor receptors, including the IGF1-R (17, 18). Amphiregulin (AR), a member of the EGF family, is a pleiotropic factor with a wide range of effects on cell proliferation, survival, and differentiation (19–21). Because its receptors, the IGF1-R, have been shown to promote survival in several cell lines (22–24), we have investigated AR induction of IGF1-R activation and its role in survival signaling.

We have shown here that the reciprocal activation of AR and IGF1 pathways induced inhibition of serum deprivation apoptosis. In NSCLC cell lines H358 and H322. We demonstrated, for the first time to our knowledge, that AR activated the IGF1-R, which in turn induced the secretion of AR and IGF1. Transactivation of the IGF1R by AR is independent of its binding to EGFR. Thus, AR can inhibit apoptosis in NSCLC cells through an IGF1-R-dependent pathway.

AR, an EGF-related growth factor produced by epithelial cells, functions as an autocrine/paracrine cell proliferation factor (42, 43). Several lines of evidence suggest that AR is an autocrine growth factor in human carcinoma cells (44–51) and cells secreted IGF1, but AR was below detectable levels (Table III).

We then used AR or IGF1 recombinant proteins to analyze inhibition of H322 cells in serum deprivation apoptosis. When AR or IGF1 were used as a single agent, the apoptosis inhibition was dose-dependant and observed only at high concentrations (Fig. 4). In contrast, when AR and IGF1 proteins were used in combination, the maximal apoptosis inhibition observed in H322 cells cultured in serum-free medium was reached within 5 ng/ml AR and 1 ng/ml IGF1, the concentrations present in H358 CM (Fig. 4). These data confirmed that both AR and IGF1 are involved in the anti-apoptotic activity of H358 CM and suggested a cooperation between the growth factors to inhibit serum deprivation apoptosis in the two cell lines.

AR Activates the IGF1-R in H322 Cells—We then analyzed the activation of the IGF1-R in H322 cells. The IGF1-R was unphosphorylated in H322 cells cultured in serum-free medium (Fig. 5A, and B). As expected, IGF1 induced the tyrosine phosphorylation of its receptor. Moreover, we observed the phosphorylation of the IGF1-R on H322 cells after 30 min of incubation in H358 CM (Fig. 5A). This phosphorylation was prevented when H358 CM was preincubated with the anti-AR neutralizing antibody (Fig. 5B). Moreover, incubation of H322 cells for 30 min with AR recombinant protein in serum-free medium induced the phosphorylation of the IGF1-R (Fig. 5B). Thus, in H322 cells, AR activated the IGF1-R. Accordingly, H358 cells cultured in serum-free medium, which secreted IGF1 and a high amount of AR, presented a constitutive phosphorylation of the IGF1-R (Fig. 5C).

Activation of the IGF1-R by AR Is Independent of the EGFR—We then analyzed whether or not the EGFR was involved in IGF1-R transactivation. Interestingly, we showed that IGF1-R phosphorylation by H358 CM or AR in H322 cells was not inhibited in the presence of the two EGFR inhibitors, AGS556 and ZD1839 (Fig. 6). Altogether, these data demonstrated that the IGF1-R can be activated by AR independently of the activation of the EGFR.

Secretions of AR and IGF1 Are Induced by the IGF1-R—The release of soluble EGF-like growth factors involves proteolysis mediated by matrix metalloproteases (40, 41) and the IGF1-R (37, 39). To determine the mechanism by which a large quantity of AR is released by H358 cells, we assessed whether treatment with the IGF1-R inhibitor AG1024 or the metalloproteases inhibitor 1,10-phenanthroline affected the AR release by serum-deprived H358 cells. As shown in Fig. 7, incubation with AG1024 or 1,10-phenanthroline both markedly decreased the AR level detected in the serum-free culture medium. In contrast, incubation of H358 cells with 1,10-phenanthroline had no effect on IGF1 level, but AG1024 decreased IGF1 release in the serum-free culture medium (Fig. 7).

The data suggested that the IGF1-R pathway regulated the AR secretion through metalloproteases activation. Furthermore, activation of the IGF1-R might also induce IGF1 release.


discussion

We have shown here that the reciprocal activation of AR and IGF1 pathways induced inhibition of serum deprivation apoptosis in NSCLC cell lines H358 and H322. We demonstrated, for the first time to our knowledge, that AR activated the IGF1-R, which in turn induced the secretion of AR and IGF1. Transactivation of the IGF1R by AR is independent of its binding to EGFR. Thus, AR can inhibit apoptosis in NSCLC cells through an IGF1-R-dependent pathway.

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The expression of EGF-related peptides and receptors in a large group of 195 stage I-IIIA NSCLC patients showed that enhanced AR expression was the only variable to be significantly correlated with a reduced overall survival (6). We confirm here that AR could be a potent inducer of NSCLC cells autonomous survival. IGF1 and its receptor the IGF1-R contribute to carcinogenesis. The IGF1-R effects several growth-promoting functions: it stimulates mitogenesis (14, 15), promotes cellular transformation (16, 18), and protects cells from apoptosis (19–33). A dominant-negative IGF1-R mutant induced extensive apoptosis in vitro and inhibited growth in vitro of rat glioblastoma C6 cells (57). We confirmed in this study that the activation of the IGF1/IGF1-R pathway inhibits NSCLC cell apoptosis.

Known EGFR ligands, including AR, are synthesized as cell surface precursor proteins, and soluble growth factors are produced by proteolysis (40, 41). Activation of the IGF1-R pathway induces the secretion of soluble EGF-related peptide such as AR and TGFα in keratinocytes and HB-EGF in COS-7 cells that in turn activate the EGFR (37, 39, 58). We showed here that inhibition of the IGF1-R or the metalloproteases on H358 cell line decreased the level of secreted AR, suggesting that the IGF1-R increased the shedding of pro-AR from H358 cell surface by metalloprotease activation, as described for HB-EGF (39). Whether or not this mechanism is sufficient to explain the very high level of AR secreted by H358 cells is under investigation. Our results showed also here that AR stimulated the IGF1-R, which in turn induced the release of AR, an amplification phenomenon that could also explain the high levels of AR secreted by H358 cells. Furthermore, H358 cells express a mutated constitutively active Ras protein (59), and Ras activation has been shown to increase the production of EGF-related peptides (60, 61). Accordingly, H322 cells, which did not secrete AR, express a wild-type Ras protein (59).

AR is known to elicit its biological activity through binding and activation of the EGFR. We demonstrated that AR can also induce the phosphorylation of IGF1-R. Furthermore, in H322 cells, the anti-apoptotic activity of H358 CM was strongly inhibited by both an IGF1-R inhibitor and anti-AR neutralizing antibody, although EGFR inhibitor and anti-IGF1 neutralizing antibody were less effective. Altogether, these data suggested that, in the presence of the two growth factors and their receptors, the anti-apoptotic effect is preferentially mediated by AR and the IGF1-R pathway. The activation of IGF1-R by EGF-related peptides has never been demonstrated, although it was suggested in results from Swantek and Baserga (36) in mouse embryo fibroblastic cells. These authors showed that EGF induced a prolonged activation of ERK2 intracellular kinase in cells containing functional IGF1-R, but only a weaker and transitory activity of the kinase in cells that do not express IGF1-R or that express a mutated form of the receptor. Transfection of cells with IGF1-R restored the capacity of EGF to induce prolonged activation of ERK2 (36), thus suggesting that the IGF1-R was required to transduce the EGF-mediated signal.

Furthermore, we showed that AR transactivates the IGF1-R independently of its binding on its specific receptor, the EGFR. Whether interaction of AR with the IGF1-R is direct or due to
oligomerization of AR and IGF1 before binding to the receptor is under investigation. Although transactivation of IGF1-R by EGFR activation has never been described before, cases of transactivation of erbB family receptors by IGF1-R have been observed (37–39). Moreover, in glioblastoma, IGF1-R signaling is involved in resistance mechanisms to anti-EGFR therapy (34), and in breast cancer cell models that overexpress erbB-2/neu, an increased level of IGF1-R signaling appears to interfere with the action of Trastuzumab, an anti-erbB2/neu antibody (35). By using combinations of antisense oligonucleotides directed against erbB-2 mRNA and IGF1-R, it has also been shown that IGF1-R directs erbB2 phosphorylation (62); this interaction involves physical association of both receptors and the formation of an heteromeric complex. Similar interaction between EGFR and IGF1-R has never been described.

Emergence of a tumoral clone resulted from accumulation of abnormalities resulting to a prolonged survival and a stimulation of growth and mobility. We previously showed that H358 NSCLC cells presented an increased resistance to p53- and BAX-mediated apoptosis (63). Resistance to apoptosis in the H358 cell line is linked to conformational abnormalities of the BAX protein and an inhibition of its translocation from the cytosol to the mitochondria; this phenomenon is reversed by treatment with caffeine. In this report, we characterized another original mechanism of apoptosis inhibition in NSCLC cells. It remains to be determined if there is a link between both these observations and, in particular, if the activation of the IGF1-R pathway by AR can modify the phosphorylation of the BCL2 family members and inactivate BAX.

In conclusion, sequestration of growth factors, in particular EGF-related peptides, by human lung epithelial cells is well established, but the consequences of the coexpression of EGF and its ligands by malignant cells on lung tumor progression are still controversial as it is for IGF1-R expression. Our study, which demonstrates for the first time the activation of IGF1-R by AR, in addition to the stimulation of AR release by IGF1-R activation, is a very important step in the elucidating the involvement of AR and IGF1 in lung tumor development and progression. Further studies on the relevance of those growth factors in vivo on fresh tumor samples will allow development of a new therapeutic strategy.

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