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IGF1 induces cell proliferation in human pituitary tumors – Functional blockade of IGF1 receptor as a novel therapeutic approach in non-functioning tumors

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ABSTRACT

Insulin-like growth factor (IGF1) and its receptor display potent proliferative and antiapoptotic activities and are considered key players in malignancy. The objective of the study was to explore the role of IGF1 and its downstream pathways in the proliferation of non-functioning pituitary tumor cells and to develop a targeted therapeutic approach for the treatment of these tumors. Cultures of human non-functioning pituitary adenomas and the non-secreting immortalized rat pituitary tumor cell line MtT/E were incubated with IGF1, IGF1 receptor inhibitor or both, and cell viability, proliferation and signaling were examined. Our results show that IGF1 elevated cell proliferation and enhanced cell cycle progression as well as the expression of cyclins D1 and D3. IGF1 also induced the phosphorylation of ERK, Akt and p70S6K. On the other hand, the selective IGF1R inhibitor NVP-AEW541 abrogated IGF1-induced cell proliferation as well as IGF1 receptor phosphorylation and downstream signaling.

1. Introduction

The involvement of the insulin-like growth factor (IGF) system in the initiation and progression of human cancer has been well established (Baserga et al., 2003; Gallagher and LeRoith, 2010; Werner and LeRoith, 1996). Activation of the IGF1 receptor (IGF1R) initiates the survival/antiapoptotic PI3K/Akt/mTOR and the mitogenic Ras/Raf/MAPK fundamental signaling cascades, with ensuing enhancement of proliferation, survival, metastasis, and angiogenesis (Baserga, 2000; Wang and Sun, 2002). Interestingly, congenital IGF1 deficiency due to growth hormone (GH) receptor dysfunction, was shown to confer a protective effect against future development of malignancies (Guevara-Aguirre et al., 2011; Steuerman et al., 2011).

The expression of IGF1R in the pituitary gland (Goodyer et al., 1984) as well as the local production of IGF1 itself, have been reported previously (Alberti et al., 1991; Bach and Bondy, 1992). IGF1 was shown to inhibit GH secretion and mRNA expression in rat pituitary cells (Morita et al., 1987) and in the rat somatotroph tumor cell line MtT/S (Niiori-Onishi et al., 1999). In addition, IGF1 was shown to suppress basal and GH releasing hormone (GHRH)-stimulated GH secretion and GH mRNA levels in human pituitary adenoma cells (Yamashita et al., 1986). Moreover, IGF1 has been shown to inhibit apoptosis in normal rat pituitary (Fernandez et al., 2004) and mouse gonadotroph cells (Rose et al., 2004) and to stimulate cell proliferation of normal mouse pituitary cells (Oomizu et al., 1998) and rat pituitary tumors (Binnerts et al., 1990). However, our knowledge regarding IGF1 effect on proliferation of human pituitary tumor cells is limited (Atkin et al., 1994, 1993; Clausen et al., 2004); the effect of IGF1 on cell proliferation was found significant in two of three non-functioning (NF) tumors tested but was not significant in the pooled results (Clausen et al., 2004). Moreover, IGF1R expression is reduced in acromegaly and does not change in NF tumors, compared to the normal pituitary, as tested by quantitative PCR and immunostaining (Kola et al., 2003; Otsuka et al., 1999).

In view of the well-established role of IGF1R in tumor development, the downregulation of IGF1R function was suggested to have a major therapeutic potential. Indeed, strategies such as IGF1R neutralizing antibodies (Olmos et al., 2010) and small-molecule

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tyrosine kinase inhibitors were found to interfere with cell growth and proliferation in multiple types of cancer, including melanoma, breast cancer, neuroendocrine tumors and others (Haisa, 2013). It has also been noted that somatostatin analogs were shown to reduce IGF1 and IGF2 secretion in prostate cancer cells whereas administration of exogenous IGF1 counteracted the inhibitory effect of these compounds on proliferation (Ruscica et al., 2010). In addition, interferon-β (IFN-β) downregulated IGF2 mRNA expression in adrenocortical carcinoma cells (van Koetsveld et al., 2006) and both IGF2 and IGF1R expression levels were reduced by type I interferons, mainly IFN-β in the human carcinoid cell line BON1 (Vitale et al., 2009). Moreover, the proapoptotic activity of IFN-β on BON1 cells was partially counteracted by the coadministration of IGF1 and IGF2 (Vitale et al., 2009). Although the effects of inhibitors targeting IGF1 downstream pathways, e.g. mTOR inhibitors, were investigated in pituitary tumor cells (Cerovac et al., 2010; Gorshtein et al., 2009; Zatelli et al., 2010), no data is currently available regarding the effects of IGF1R inhibitors on these cells.

The major treatment strategy for NF pituitary tumors is pituitary surgery, even though post-operative recurrence rates are as high as 50%. This being the case and that no efficient adjuvant medical treatment is available, we aimed to examine the involvement of IGF1 in the propagation of NF pituitary tumor cells and to develop a selective IGF1R targeted therapeutic approach for the treatment of these tumors.

2. Materials and methods

2.1. Reagents

Antibodies against total IGF1R (#3027, Rabbit), p70/S6K (#9202, Rabbit), pp70/S6K-Thr389 (#9234, Rabbit), total Akt (#4691, Rabbit), pAkt-Ser473 (#9271, Rabbit), cyclin D1 (#2926, Mouse), cyclin D3 (#2936, Mouse), cyclin E (#4129, Mouse) and CDK2 (#2546, Rabbit) were obtained from Cell Signaling Technology (Beverly, MA, USA). All antibodies were diluted 1:1000 in 5% non-fat dry milk in Tris-buffered saline (TBS)/Tween 20. Antibodies against total ERK (M5670, Rabbit), pERK (M8159, Mouse) and β-actin (A5441, Mouse) were from Sigma–Aldrich Ltd. (Israel) and were diluted 1:10000 in 5% non-fat dry milk in TBS/Tween 20. Horseradish peroxidase-conjugated secondary antibodies were purchased from DAKO (Glostrup, Denmark). IGF1 was purchased from PeproTech Asia (Rehovot, Israel). NVP-AEW541 is a pyrrolo [2,3-d]pyrimidine derivative selective IGF1R tyrosine kinase inhibitor. NVP-AEW541 is capable of distinguishing between the IGF1R (IC_{50} = 0.086 μM) and the closely related insulin receptor (InsR) (IC_{50} = 2.3 μM) in cells (Garcia-Echeverria et al., 2004). It was kindly provided by Novartis Pharma AG (Basel, Switzerland) and was dissolved in DMSO.

2.2. Cell cultures and pituitary tumors

MtT/E cells (a rat pituitary tumor cell line, kindly provided by Dr. M. Theodoropoulou, Max Planck Institute of Psychiatry, Munich, Germany) were cultured in high glucose DMEM supplemented with 10% FCS, 2 mM glutamine, 12.5 U/ml nystatin, 0.1 mg/ml streptomycin and 100 U/ml penicillin, at 37 °C and 5% CO_2. Samples of pituitary adenomas were obtained during curative transsphenoidal surgical resection with informed consent, in accordance with methods and conditions approved by the local Institutional Review Board. Altogether 32 non-functioning macroadenomas (NFs) were included. The clinical characteristics of the adenomas are presented in Table 1. Specimens were mechanically dispersed and enzymatically dissociated using 0.35% collagenase and 0.1% hyaluronidase, as previously described (Rubinfeld et al., 2006) and were cultured in low glucose DMEM supplemented with 10% FCS.

2.3. Cell viability

For cell viability assays, NF cells (~5×10^4/well) and MtT/E cells (~1×10^4/well) were seeded in 96-well tissue culture plates. Two days later, cells were treated with 50 ng/ml IGF1 or NVP-AEW541 (1 μM except for Fig. 2A right-0.1, 0.3, 1 and 3 μM) or both for 48 h in starvation medium. DMSO served as control in experiments with NVP-AEW541. Untreated cells were used as controls in experiments with only IGF1. Cell viability was measured using a Cell Proliferation Assay with XTT reagent (Biological Industries, Beit-Haemek, Israel), according to kit instructions. All assays were performed in six replicates. Cell viability of three NFs (NF19, NF28, NF30) was also examined in MEM medium where D-valine was substituted for L-valine (Biological Industries) to inhibit fibroblasts proliferation. Cell viability of MtT/E cells was also measured using the Hoechst assay (McCaffrey et al., 1988) with a slight modification as subsequently described. MtT/E cells (~1×10^4/well) were seeded in tissue culture 96-well plates for 24 h, and then exposed to IGF1 as indicated or 1 μM NVP-AEW541 or both in starvation medium (in six replicates). After 48 h the medium was discarded and cells were fixed with ethanol (70%) for 30 min. The ethanol was discarded and 200 μl of 10 μg/ml bis-benzimide Hoechst fluorescent probe #B-2261 (Sigma–Aldrich Ltd.) solubilized in PBS was added and the fluorescence was measured at 390 nm with a FluoStar fluorometer.

2.4. Cell proliferation

MtT/E cells (~1×10^4/well) were seeded in 96-well plates. The following day cells were treated with 50 ng/ml IGF1 or 1 μM NVP-AEW541 or both in starvation medium for 48 h. BrdU assay was conducted according to manufacturer's instructions (BrdU Cell Proliferation Assay, Calbiochem, La Jolla, CA, USA). Briefly, the BrdU label was added to cultures and allowed to incubate at 37 °C for 6 h. At the end of the incubation period, the medium was removed and the cells were fixed for 30 min at room temperature. Anti-BrdU antibody was then added for 60 min and cells were washed with washing buffer. Peroxidase Goat Anti-Mouse IgG HRP Conjugate was added for 30 min and cells were washed with washing buffer and distilled H_2O. Substrate solution was added for 15 min and stop solution was added to complete the reaction. Absorbance was measured at 450 nm.

2.5. Cell cycle analysis

Cell cycle was analyzed by flow cytometry after propidium iodide staining. MtT/E cells were plated in 6 cm plates and subjected to the indicated treatments for 48 h. Cells were scraped with a rubber policeman, washed with PBS, and fixed in 70% ethanol for 30 min or overnight at minus 20 °C. Cells were then washed with PBS and resuspended in staining buffer containing propidium iodide (50 μg/ml), RNase A (100 μg/ml) and 0.1% Triton X-100. Analysis was done in a fluorescence-activated cell sorter, FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA), using CellQuest and ModFit Software.

2.6. Protein extraction and Western blotting

For IGF1R and cyclin D1 detection in NF tissue samples, frozen tissue specimens were minced in liquid nitrogen followed by homogenization in RIPA buffer. For the assessment of activated protein levels of ERK, Akt and p70S6K in response to treatment, MtT/E and NF cells were serum-starved (0.1% FCS) for 20–24 h.

Table 1
Clinical characteristics of patients with non-functioning pituitary adenomas and experiments performed.

Tissue type	Size (mm)	Significant CS invasion	Gender/age (yr)	Immunostaining	XTT	Western
NF1	20	Lt	F/55	FSH + focal PRL	IGF1*	–
NF2	25	Rt	F/60	FSH + β-LH	IGF1*	–
NF3	27	NA	F/58	Focal FSH, β-LH	IGF1*	–
NF4	26	Rt	M/76	Focal FSH, β-LH	IGF1*	–
NF5	30	Lt	M/59	Negative	IGF1*	–
NF6	17	Rt	F/65	FSH + β-LH	IGF1	–
NF7	30	None	M/46	Negative	IGF1	–
NF8	35	Bilateral	M/54	Focal FSH, β-LH	IGF1	–
NF9	22	NA	M/38	Focal FSH, β-LH	IGF1	–
NF10	35	Lt	F/36	ACTH	IGF1*	–
NF11	22	Rt	M/69	Negative	IGF1*	–
NF12	20	Rt	F/84	FSH + β-LH	IGF1	–
NF13	22	Rt	M/61	Negative	IGF1*	–
NF14	23	Lt	F/68	Focal FSH, β-LH	IGF1*	–
NF15	50	Lt	M/38	Focal FSH, ACTH	IGF1*, AEW, IGF1 + AEW	–
NF16	40	None	F/33	Focal FSH	IGF1*, AEW, IGF1 + AEW	–
NF17	44	None	M/58	Focal FSH, scattered β-LH	IGF1, AEW	+
NF18	20	None	M/84	Focal FSH, β-LH	IGF1, AEW	–
NF19	22	None	F/35	ACTH	IGF1*, AEW, IGF1 + AEW	+
NF20	18	Lt	M/54	Negative	IGF1*, AEW, IGF1 + AEW	–
NF21	20	None	M/35	FSH + β-LH	IGF1*, AEW	–
NF22	30	Rt	M/58	Scattered FSH, rare β-LH	IGF1*, AEW	+
NF23	24	Rt	M/52	FSH	IGF1, AEW	–
NF24	18	None	M/55	FSH + β-LH	IGF1*, AEW	+
NF25	33	None	M/70	FSH + β-LH	IGF1*, AEW	–
NF26	28	Rt	M/80	FSH + β-LH	IGF1	+
NF27	28	Lt	M/60	FSH + β-LH	IGF1	+
NF28	14	None	M/66	FSH + β-LH	IGF1*, AEW, IGF1+AEW	+
NF29	26	Rt	M/73	FSH + β-LH	IGF1	+
NF30	21	Lt	M/60	FSH + β-LH	IGF1*, AEW, IGF1 + AEW	–
NF31	37	Rt	M/41	Focal FSH	–	+
NF32	Macro	Rt	M/67	FSH	–	+

M, male; F, female; CS, cavernous sinus; Lt, left; Rt, right.
* Significant viability elevation.

IGF1 (50 ng/ml) was then added to the medium for 10 min followed by treatment with 1 μM NVP-AEW541 for 30 min. To assess cell cycle progression, we used cyclin D1, cyclin D3, cyclin E and CDK2 detection in cells treated with IGF1 and NVP-AEW541 for 48 h. DMSO was used as a control in the relevant experiments. For all detections lysates were prepared using RIPA buffer supplemented with protease and phosphatase inhibitors cocktails, all from Sigma–Aldrich Ltd. Equal protein aliquots and the same amounts for the detection of total and phosphorylated proteins were loaded on 10% SDS–PAGE, transferred into nitrocellulose membranes, blocked and incubated overnight at 4 °C with the appropriate antibodies. After 3 washes in TBS/Tween 20, the membranes were incubated with the secondary antibody for 60 min. Immunodetection was performed using the Chemiluminescent Peroxidase Substrate (Sigma–Aldrich Ltd.). In the case of MtT/E cells, each assay was repeated with lysates obtained from three independent experiments. Optical density of the bands was measured employing the VersaDoc Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA).

2.7. Statistical analysis

Results were expressed as mean ± SE. Data were analyzed by one-way ANOVA (StatView software, SAS Institute Inc., NC, USA), and P values less than 0.05 were considered significant.

3. Results

3.1. Effect of NVP-AEW541 on the IGF1-stimulated viability of human non-functioning pituitary tumor cells

Incubation of cells from 30 non-functioning pituitary tumors with IGF1 (50 ng/ml) in starvation medium for 48 h, significantly stimulated cell viability by 20–40% in 20 tumors (Fig. 1A, left). Western blotting was applied to assess IGF1R expression levels in four responding and four nonresponding NFs and no correlation in the response to IGF1 and receptor expression was found (Fig. 1A, right). Treatment of cells derived from 6 non-functioning responding pituitary tumors (NF15, NF16, NF19, NF20, NF28, NF30) with the IGF1R inhibitor, NVP-AEW541, significantly abrogated the IGF1-induced cell viability in pooled tested tumors (Fig. 1B) and in each individual tumor (Fig. 1C). Since adenoma cell cultures are contaminated with fibroblasts, it was essential to confirm that the effects of IGF1 and NVP-AEW541 on viable cell numbers were not due to fibroblast growth. Therefore, cell viability of three NFs (NF19, NF28, NF30) was examined in cultures maintained either in D-val MEM (which inhibits fibroblast growth) or in DMEM, following the protocol described in Section 2. Results obtained showed that the effects of IGF1 and NVP-AEW541 on cell viability were similar under both growth conditions (Fig. 1C). It is of note that NVP-AEW541 reduced basal cell viability in only 4 out of 13 NFs tested, induced cell viability in 3 of 13 and 6 were insignificantly affected (Fig. 1D). No correlation was found between clinical characteristics including tumor size or cavernous sinus invasion, with viability response to IGF1 or NVP-AEW541.

3.2. Effect of NVP-AEW541 on the IGF1-enhanced proliferation of MtT/E cells

MtT/E cells were incubated with IGF1, NVP-AEW541 or both, and 48 h later their effect on cell viability was measured by the XTT and Hoechst assays. Treatment with IGF1 (5, 20, 50 and 100 ng/ml) resulted in a significant elevation in the number of viable cells (Hoechst test), reaching its maximal effect at 20 and 50 ng/ml concentrations (Fig. 2A, Left). The effect of 50 ng/ml IGF1 was also found significant in XTT assays (Fig. 2A, right) and

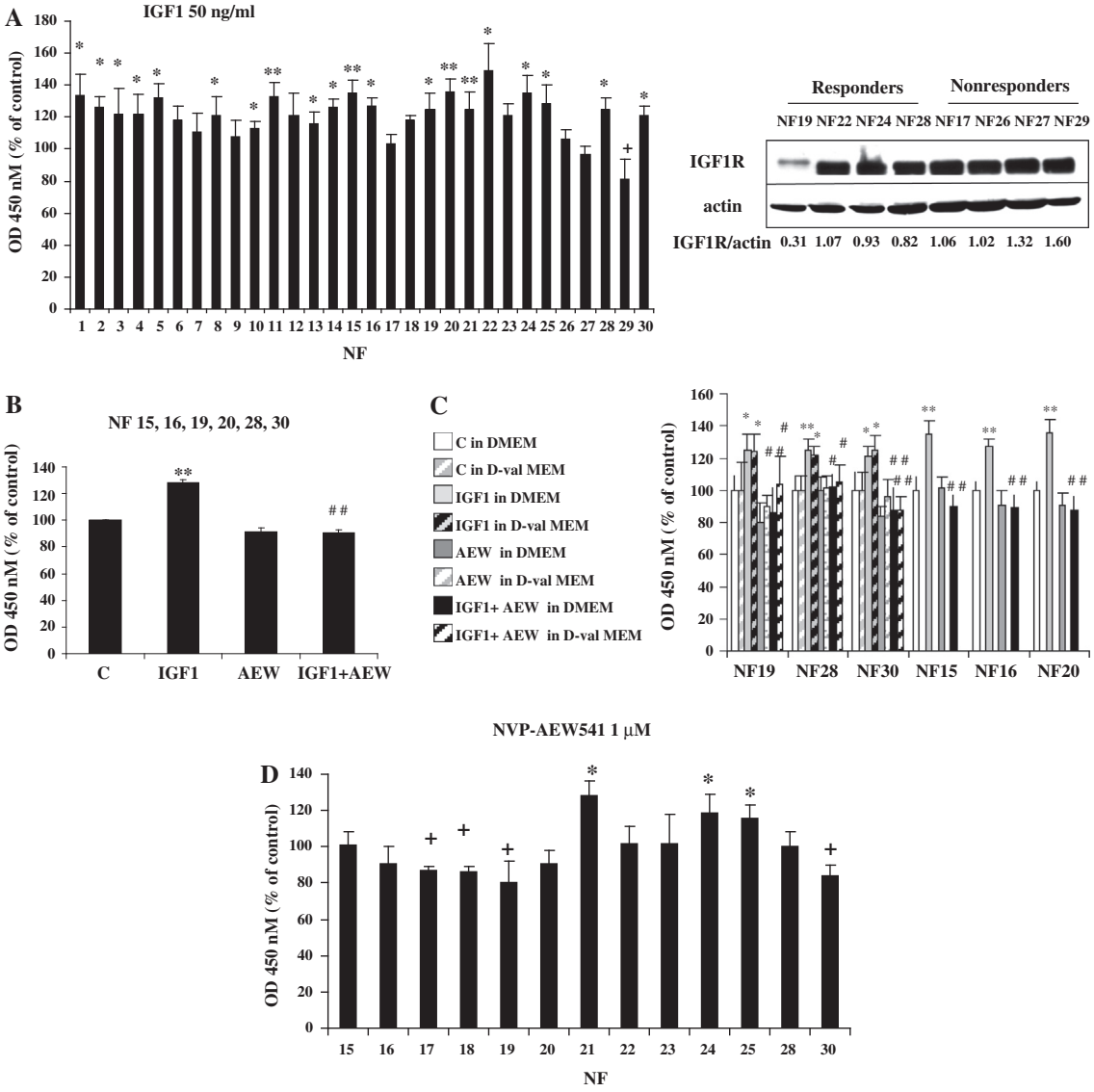

Fig. 1. Effect of IGF1 and IGF1R inhibitor on cell viability of human NFs. (A) Left: effect of IGF1 (50 ng/ml) on proliferation of individual NFs ($n = 30$). (A) Right: expression level of IGF1R in responder and nonresponder NFs. Equal protein loading was examined by the detection of β-actin. Densitometric values of IGF1R/β-actin ratio are indicated below the blot. (B) NFs 15, 16, 19, 20, 28 and 30 were incubated with IGF1 (50 ng/ml), NVP-AEW541 (1 μM) or both. (C) NFs 19, 28, 30 were incubated with IGF1 (50 ng/ml), NVP-AEW541 (1 μM) or both in D-val MEM or DMEM. NFs 15, 16, 20 were incubated with IGF1 (50 ng/ml), NVP-AEW541 (1 μM) or both in DMEM. (D) Thirteen NFs were incubated with NVP-AEW541 (1 μM). All treatments (panels A, B, C, D) were performed in starvation medium for 48 h. Cell viability was examined using a cell proliferation assay with XTT reagent. (B) Values are expressed as the average and S.E.M. of 6 adenomas. (A, C, D) Values are expressed as average and S.D. of six wells compared with control wells (100%). (A) Control wells were untreated. (B-D) Control wells were treated with 10^{-3} DMSO. $^{*}P < 0.05$ increase versus control, $^{**}P < 0.0001$ increase versus control, $^{\#}P < 0.05$ decrease versus IGF1-treated cells, $^{\#\#}P < 0.0001$ decrease versus IGF1-treated cells, $^{+}P < 0.05$ decrease versus control.

was used in all further experiments. Treatment of MtT/E cells with NVP-AEW541 resulted in a significant decrease in the number of IGF1-induced viable cells at concentrations of 0.3, 1 and 3 μM (Fig. 2A, right). Significant reduction of IGF1 induction with a concentration of 1 μM NVP-AEW541 was confirmed by the Hoechst assay (Fig. 2B) and was used in all further experiments. BrdU experiments were performed in order to verify the effects of IGF1 and NVP-AEW541 on the proliferation of MtT/E cells. Incubation with IGF1 resulted in a marked induction of BrdU incorporation that was significantly inhibited by NVP-AEW541 (Fig. 2C).

3.3. Effect of NVP-AEW541 on the IGF1-facilitated ERK, Akt and p70S6K phosphorylation

We next assessed the effect of IGF1 on the phosphorylation of ERK, Akt and p70S6K in MtT/E cells. IGF1 induced robust phosphorylation of Akt and significantly increased also the phosphorylation of ERK and p70S6K (Fig. 3A). Moreover, marked elevations of ERK, Akt and p70S6K phosphorylations were observed after the treatment of cultured NFs tumors (NF31 and NF32) with IGF1 (Fig. 3B). Incubation with NVP-AEW541 significantly reduced the IGF1-induced phosphorylations in all tested cells (Fig. 3A and B).

3.4. Effect of NVP-AEW541 on the IGF1-enhanced cell cycle progression and cyclins D1 and D3 protein levels

We next examined whether the enhanced proliferative effect of IGF1 in MtT/E cells reflects cell cycle acceleration. MtT/E cells were incubated with IGF1, NVP-AEW541 or both, and 48 h later were analyzed by flow cytometry. The proportion of cells in the S-phase was significantly elevated by IGF1 (50%; Fig. 4A) and was reduced to control levels by NVP-AEW541 (Fig. 4A). Western blot analysis

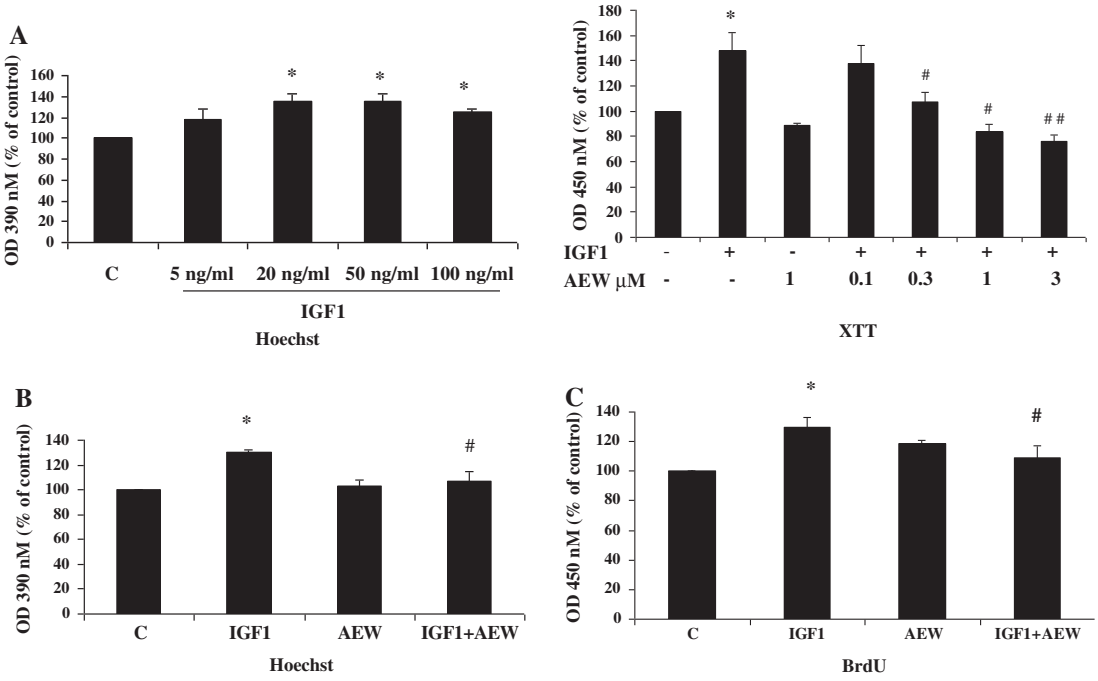

Fig. 2. Effect of IGF1 and IGF1R inhibitor on cell viability and proliferation of MtT/E cells. (A) Left: MtT/E cells were incubated with or without (control) the indicated concentrations of IGF1 and viability was determined by the Hoechst test. (A) Right: MtT/E cells were incubated with 50 ng/ml IGF1, with or without the indicated concentrations of NVP-AEW541 and viability was determined by the XTT assay. (B and C) MtT/E cells were incubated with DMSO (10^{-3}, control), IGF1 (50 ng/ml), NVP-AEW541 (1 μM) or both. (B) Viability was determined by the Hoechst test. (C) BrdU incorporation was examined using the BrdU Cell Proliferation Assay (Calbiochem). Values are expressed as average and S.E.M. of three independent experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus IGF1-treated cells. $^{\#\#}P < 0.0001$ versus IGF1-treated cells.

revealed elevations in cyclins D1 and D3 protein levels following treatment with IGF1 (Fig. 4B). Treatment with NVP-AEW541 reversed the IGF1-induced expressions of cyclins D1 and D3 to the control levels (Fig. 4B). Cyclin E and CDK2 protein levels were not influenced by IGF1 or NVP-AEW541 (Fig. 4B). We next assessed the expression of cyclin D1 in the IGF1-responding and nonresponding NF tumors. Interestingly, the expression of cyclin D1 was significantly higher in the four nonresponding, compared with the responding NFs (Fig. 4C). The mean cyclin D1 levels (normalized to actin) were 3-fold higher in the nonresponders versus responders NFs (Fig. 4C). The effect of NVP-AEW541 on cell viability was tested in only one of the cyclin D1 highly expressing NFs, therefore correlation cannot be examined.

4. Discussion

The present study demonstrates for the first time the effects of IGF1R inhibition on human pituitary non-functioning tumors *in vitro*. Our results indicate that IGF1 induces cell viability of human pituitary tumor cells derived from non-functioning adenomas. By applying XTT and Hoechst assays (which measure viability parameters by metabolic activity and nucleic acids staining respectively), BrdU assays (which measure nucleotide incorporation into replicating DNA), and FACS analyses, we were able to demonstrate and validate that IGF1 induces cell cycle progression in the MtT/E cell line, with ensuing pituitary tumor cell proliferation. This was further confirmed by the elevation in cyclins D1 and D3 expression that facilitate G1/S transition, the major although not the only cell cycle checkpoint positively regulated by IGF1 (Samani et al., 2007). Cyclin E and CDK2 expression levels were not affected by IGF1, suggesting that IGF1 regulates early, not late G1-phase events; although it was examined in MtT/E cells and IGF1 may act differently in human pituitary NF tumors. Our results also show IGF1 induction of PI3K/Akt and ERK signaling pathways that are known to mediate IGF1R activation (Shelton et al., 2004), in both human pituitary tumor cells and MtT/E cells.

The increased cell viability of 20 out of 30 (67%) NF tumors exposed to IGF1 in this study is in line with the findings of previous studies showing the increased cell viability in five of seven (71%) gonadotropin-secreting tumors (Atkin et al., 1993) and two of three (67%) NF tumors after IGF1 treatment (Clausen et al., 2004). Also, IGF1 was shown to promote cell viability by about 42% in a group of the mTOR inhibitor (RAD001) responder NFs (Zatelli et al., 2010). Although, IGF1 significantly stimulated the proliferation of corticotrophs and mammotrophs in mouse normal pituitary cells, this did not occur with thyrotrophs, gonadotrophs or somatotrophs (Oomizu et al., 1998). Increased proliferation of prolactin-secreting cells by IGF1 *in vitro* was also found in rat pituitary tumor cells originating from a transplanted tumor (Binnerts et al., 1990), and in one prolactinoma out of two human somatotrophinomas and two prolactinomas studied (Atkin et al., 1994). Thus, proliferative responses to IGF1 seem to vary among the various pituitary cell types, although for some human pituitary tumor types information is still missing or the number of tumors studied was small. This differential response to IGF1 may be attributed to IGF1R expression levels. Studies in human samples showed that normal pituitaries and tumors arising from somatotroph, corticotroph, lactotroph and gonadotroph cells, as well as nonfunctioning adenomas, express the IGF1R (Kola et al., 2003; Otsuka et al., 1999; Beuschlein et al., 2005). IGF1R mRNA levels as well as the percentage of immunostained positive cells were significantly lower in somatotroph tumors compared to the normal pituitary, while the other tumor types showed no significant differences from the normal pituitary (Kola et al., 2003; Otsuka et al., 1999). In our study, IGF1R expression levels were tested in representative IGF1-responding and nonresponding adenomas and, in general, were found similar (with the exception of the lower expression in the IGF1-responding NF19) with no correlation to IGF1-induced

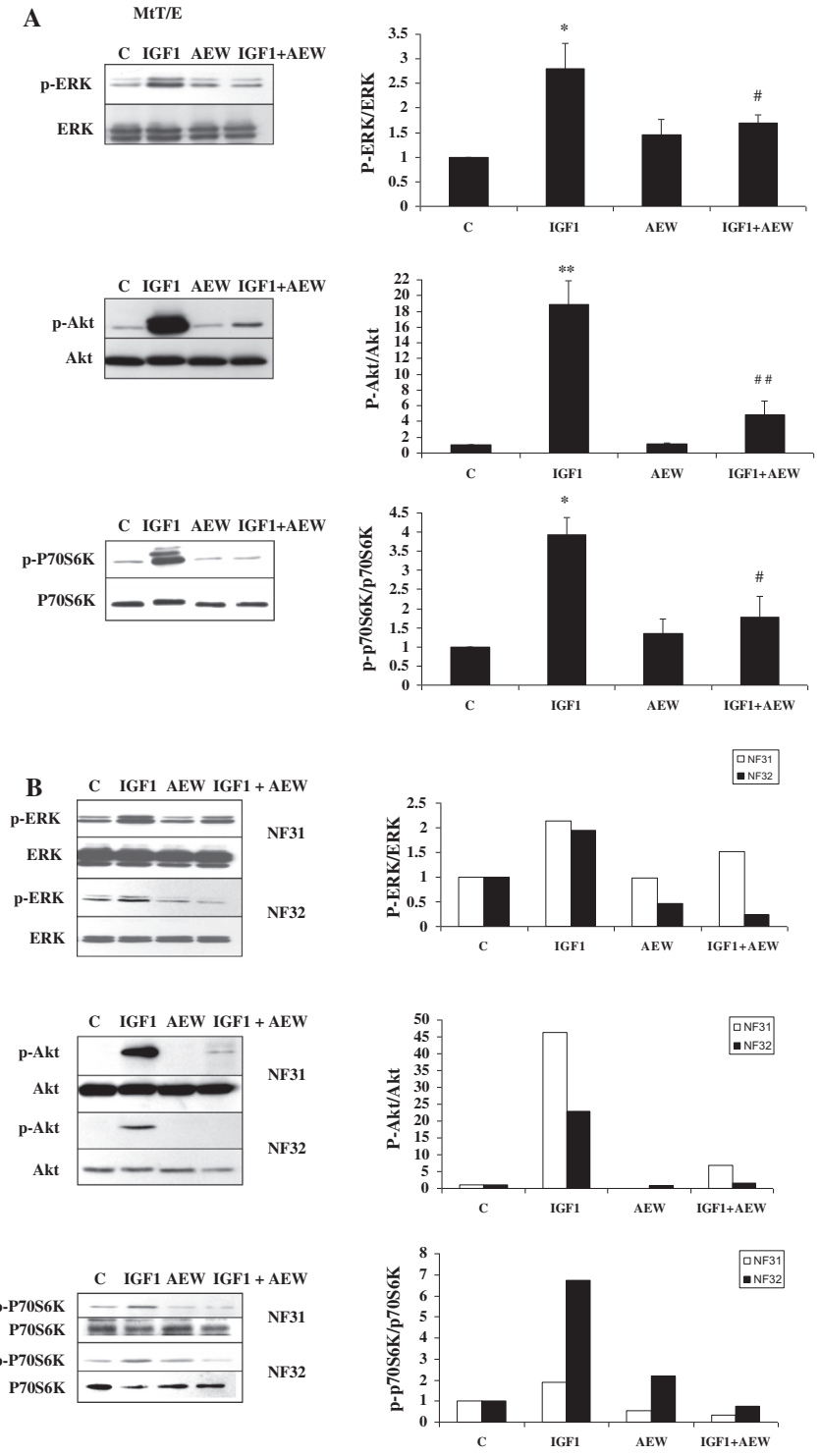

Fig. 3. Effect of IGF1 and IGF1R inhibitor on ERK, Akt and p70S6K phosphorylation. MtT/E cells (A) and NF cells were (B) serum-starved for 16–24 h and then incubated with DMSO (10^{-3}, control), IGF1 (50 ng/ml), NVP-AEW541 (1 μM) or both. Cells were treated with NVP-AEW for 30 min, after which IGF1 was added for 10 min. Levels of phosphorylated and total ERK, Akt and p70S6K were examined by Western blot analysis. (A) For each protein, a representative blot out of three independent experiments is shown. The bars represent mean + SE densitometric values of phospho/total protein ratio of three experiments. $^{*}P < 0.05$ versus control, $^{**}P < 0.0001$ versus control, $^{\#}P < 0.05$ versus cells treated with IGF1, $^{\#\#}P < 0.0001$ versus cells treated with IGF1. (B) Two NF tumors are shown and quantified.

response. Thus, overall, IGF1R expression does not seem to necessarily ensure a significant proliferative response to IGF1, suggesting the involvement of other factors affecting the functional expression of IGF1R.

Alterations in one or more regulators of the G1/S transition seem to be a frequent event (80%) in pituitary tumors (Simpson et al., 2001). Moreover, cyclin D1 overexpression is observed more frequently in nonfunctional tumors compared with other tumor types or the normal pituitary (Hibberts et al., 1999; Jordan et al., 2000; Turner et al., 2000). Therefore, we undertook to measure the expression of cyclin D1 in the NF adenomas. We found that the correlation between the response to IGF1 and cyclin D1

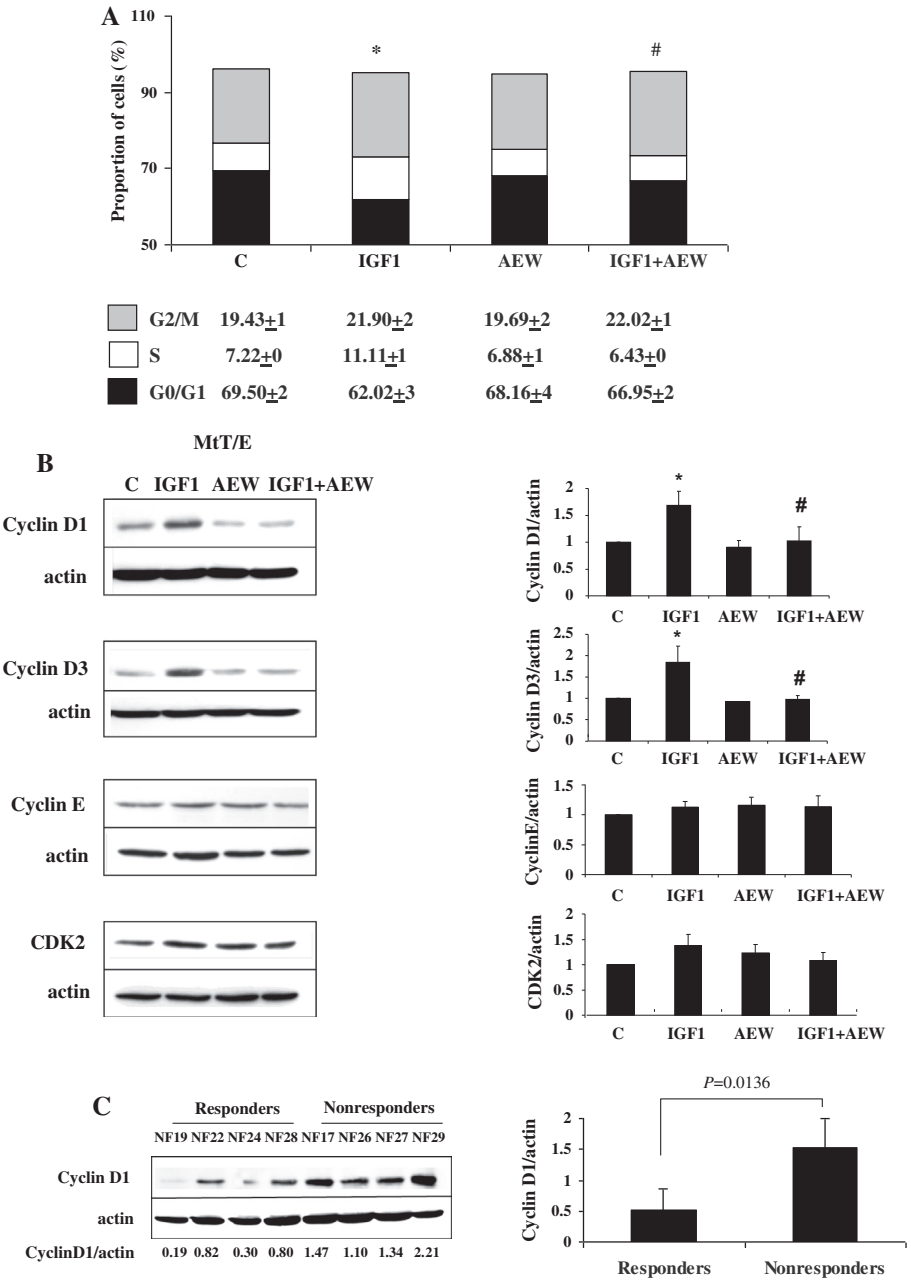

Fig. 4. Effect of IGF1 and IGF1R inhibitor on the cell cycle progression and the regulators involved. MtT/E cells were incubated with DMSO (10^{-3}, control), IGF1 (50 ng/ml), NVP-AEW541 (1 μM) or both in starvation medium for 48 h. (A) Cells were analyzed by flow cytometry. B- Protein expression levels of cyclins D1, D3 and E and CDK2 were examined by Western blot analysis. For each protein, a representative blot out of three performed is shown. The bars represent mean + SE densitometric values of three experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus cells treated with IGF1. (C) Protein expression level of cyclin D1 in NFs was examined by Western blot analysis. Equal protein loading was examined by the detection of β-actin. Densitometric values of cyclin D1/β-actin ratio are indicated below the blot and are shown as mean + SE in graph.

expression in the representative NF adenomas tested was reversed (Fig. 4C); i.e., the expression of cyclin D1 was significantly higher in the nonresponding, compared with the responding NFs. An explanation for these results could be that in the responding NFs, IGF1 increases cyclin D1 expression (as shown in the MtT/E cells, Fig. 4B), thus inducing cell cycle progression as reflected by the increased number of viable cells. However, an overactivated IGF1 autocrine circuit in the cyclin D1 overexpressing NFs reaches a steady state that prevents any response to an external IGF1. The cause of the increased cyclin D1 expression in NFs is still unknown. No correlation between the overexpression of cyclin D1 and CCND1 allelic amplification or 11q13 rearrangement in pituitary tumors have been observed (Hibberts et al., 1999; Metzger et al., 1999). Increased cyclin D1 expression was not significantly associated with the expression levels of other components of the G1/S transition, pRb or p16 (Simpson et al., 2001). An increase in growth factors acting as mitogenic stimuli leading to increased cyclin D1 expression is an alternative possibility.

The increased proliferation of pituitary tumor cells in response to IGF1 might be relevant to the safety of GH replacement therapy in GH deficient adults with a history of pituitary adenoma. All studies addressing this issue so far, including a study of large cohorts of NF patients receiving (121 patients) or not receiving (114 patients) long-term GH replacement (Olsson et al., 2009), reported that GH substitution does not increase the risk of tumor progression or recurrence (Olsson et al., 2009; Arnold et al.,

2009; Buchfelder et al., 2007; Chung et al., 2008; Frajese et al., 2001; Hatrick et al., 2002). Moreover, according to a recently published report based on the KIMS study, GH replacement in a large cohort of hypopituitary subjects may be considered a safe treatment (Gaillard et al., 2012). However, as these patients are treated with GH regimens maintaining serum IGF1 within the age-related reference range and not beyond, the in vivo effects of supra-physiological doses of IGF1 on the progression and recurrence of pituitary adenomas are not relevant to these patients.

IGF1R inhibition by the small molecule inhibitor NVP-AEW541 abrogated IGF1-induced activities, namely, cell viability, cell proliferation, phosphorylation of ERK, Akt and p70S6K, cell cycle progression and expression of cyclins D1 and D3 in human pituitary tumor cells and MtT/E cells. These findings are consistent with other reports demonstrating NVP-AEW541 effects in various tumors *in vitro* (reviewed in Haisa (2013)). Regarding the potency of inhibitors targeting the IGF1R/PI3K/Akt/mTOR pathway at basal, non-induced conditions, although the mTOR inhibitor, RAD001, inhibited pituitary cell proliferation in dispersed GH-adenomas *in vitro* (Gorshtein et al., 2009), partial response was reported in NFs treated with everolimus or rapamycin (Cerovac et al., 2010; Zatelli et al., 2010). At basal conditions a limited response is now shown also for NFs treated with an IGF1R inhibitor with significantly reduced cell viability in 4 out of 13 tested tumors. It is also important to mention that unexpectedly, elevation was induced in 3 tumors, a point which should be further examined in a larger cohort of tumors (Fig. 1D). Feedback activation of ERK or other components of EGFR signaling cascade have already been reported in various cells, and thus provide the rationale for cotargeting of IGFR and EGFR (Buck et al., 2008; Desbois-Mouthon et al., 2009; Huang et al., 2009; Nolting et al., 2012). Other dual inhibitions either by combined treatments or by one compound targeting two signaling components were shown also to improve the anti-proliferative outcome in dispersed NFs (Cerovac et al., 2010; Zatelli et al., 2010; Lee et al., 2011). Hence, data may suggest that blocking feedback loops by dual inhibition rather than specificity of only one inhibited signaling component confers higher sensitivity. This hypothesis should be further examined. In summary, our results suggest that the IGF1R may constitute a molecular target, alone or in combination with other component/s of the PI3K/Akt/mTOR signaling cascade, for efficient anti-proliferation therapy of patients with invasive non-functioning pituitary tumors. As these tumors usually do not respond to any available pituitary-targeted medical treatment, this potentially alternative option for treating patients harboring invasive adenomas should be extensively studied.

Disclosure summary

The authors have nothing to disclose.

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