Expression of activin A and its receptors in human pheochromocytomas

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Abstract

Activin A (a homodimer of two activin β A subunits) has been shown to induce the neuronal differentiation of rat pheochromocytoma PC12 cells. We studied activin A and its receptor gene expression in human pheochromocytomas *in vivo* and *in vitro* to clarify the potential involvement of activin A in the pathophysiology of these tumors. We first screened 20 pheochromocytomas and nine normal adrenal tissues for activin β A mRNA expression. Northern blots hybridized with specific oligonucleotide probes detected weak signals for activin β A transcripts in pheochromocytomas. Both type I and type II activin receptor (ActR-I, ActR-IB and ActR-II) mRNA expression was also detectable in the pheochromocytoma tissues.

In primary cultures of pheochromocytoma cells, expression of activin β A mRNA was readily detectable by Northern blotting, and secretion of activin A into the conditioned medium was confirmed by an enzyme-linked immunosorbent assay. The expression of activin β A mRNA and secretion of activin A were induced by

(Bu)₂cAMP after 1 and 3 days of treatment (all $P < 0.05$). A protein kinase inhibitor, staurosporine, inhibited the basal and (Bu)₂cAMP-induced accumulation of activin β A mRNA ($P < 0.05$). In addition, induction of chromaffin phenotype by dexamethasone also inhibited the basal and (Bu)₂cAMP-induced expression of activin A at both mRNA and protein levels (all $P < 0.05$). In contrast, the expression of ActR-I and ActR-IB mRNAs was not affected by these agents in cultured pheochromocytoma cells.

In summary, activin β A subunit and activin receptors are expressed in human pheochromocytomas. Production of activin A in cultured pheochromocytoma cells is induced through the protein kinase A pathway, but reduced during chromaffin differentiation. Therefore, activin A may function as a local neurotrophic factor via an auto/paracrine manner in human pheochromocytomas.

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Introduction

Activins were initially purified from ovarian fluids as modulators of follicle-stimulating hormone secretion in the anterior pituitary gland. They are synthesized as homo- or heterodimers of two highly related β -subunits (β A and β B), resulting in three possible molecular species, activin A (β A β A), activin AB (β A β B), and activin B (β B β B). Activins have been shown to signal via hetero-oligomers of the type I (ActR-I and ActR-IB) and type II (ActR-II and ActR-IIB) receptors (Woodruff 1998). Recently, the role of activins in the nervous system has aroused increasing interest as there is accumulating evidence for a role of activin in a number of different neural functions (Ebendal *et al.* 1998). Activin β A mRNA is expressed in the rat brain, and its levels are regulated by physiological excitatory synaptic activity and developmental signals (Andreasson & Worley 1995). The mRNAs of both type

I and type II activin receptors have been observed in neuronal cells throughout the brain (Morita *et al.* 1996). Activin A is a potent survival and neurotrophic factor for different neuronal cells (Schubert *et al.* 1990, Iwahori *et al.* 1997, Trudeau *et al.* 1997).

Activin A has been shown to induce the neuronal differentiation of rat pheochromocytoma PC12 cells. It is able to increase neurite outgrowth and some neuronal gene expression, and to suppress proliferation of PC12 cells (Paralkar *et al.* 1992, Hashimoto *et al.* 1993, Iwasaki *et al.* 1996). These reports raise the possibility that activin A may have a role in the development and/or the hormonal activity of pheochromocytomas. In human adrenals, expression of activin β A mRNA was detected in the cortical cells, and it was spontaneously induced in primary cultures (Voutilainen *et al.* 1991). To further clarify the possible involvement of activin A in the pathophysiology of pheochromocytomas, we investigated the expression of

activin and its receptor genes in human pheochromocytomas, and studied the regulation patterns of activin gene expression in cultured human pheochromocytoma cells. Our results indicate that activin A may function as a local neurotrophic factor via an auto/paracrine manner in human pheochromocytomas.

Materials and Methods

Tissues and cell cultures

Normal adrenal glands were obtained from nine patients who underwent nephrectomy for kidney tumors. Pheochromocytomas and adrenal tissues adjacent to the tumors were obtained from 20 patients during operations performed at the Department of Surgery, Helsinki University Central Hospital. The pheochromocytomas studied included 17 intra-adrenal benign tumors, two intra-adrenal malignant tumors, and one extra-adrenal benign tumor. The tissue samples were processed as described previously (Liu *et al.* 1994). Briefly, part of the normal adrenal and pheochromocytoma tissues was frozen in liquid nitrogen and then stored at -70°C until extraction of total RNA. The remaining pheochromocytoma tissues were prepared for primary cultures. All experiments were performed in triplicate and repeated at least twice with tissues from different patients.

RNA analysis

Isolation of total and cytoplasmic RNA, Northern blotting, and hybridizations were carried out as described previously (Voutilainen *et al.* 1986, Liu *et al.* 1994). For detecting activin βA mRNA expression, an oligonucleotide complementary to human activin βA mRNA (Voutilainen *et al.* 1991) was used for hybridization. Since the signals were very weak, an additional oligonucleotide was synthesized. Its sequence was 5'-GCC AGA GGG AGC AAT GAT CCA GTC ATT CCA GCC-3', corresponding to nucleotides 1236–1268 of the human activin βA mRNA (GenBank accession no. M13436; Mason *et al.* 1986). These two oligonucleotides were labeled simultaneously, and used together for hybridization. All activin βA mRNA data shown here are from Northern blots hybridized with these two oligonucleotides.

For detecting the expression of activin receptor mRNAs, specific oligonucleotides were used as probes for ActR-I and ActR-IB. The sequence of the ActR-I specific oligonucleotide probe was 5'-ATG CAT GAC TGC CAG GCC CAA ATC TGC TAT-3', corresponding to nucleotides 1157–1186 of the mRNA (GenBank accession no. Z22534, ten Dijke *et al.* 1993). The ActR-IB specific oligonucleotide sequence was 5'-GTC TTT GGA GAG ACA CAT CTC ACA TGA GGG-3', complementary to nucleotides 499–528 of the mRNA

(GenBank accession no. Z22536; ten Dijke *et al.* 1993). The mouse 28S ribosomal RNA cDNA probe (Arnheim 1979) was used to control RNA loading. The relative intensities of autoradiographic signals were quantitated by densitometric scanning. All mRNA data shown were normalized with the respective 28S RNA values.

Activin A assay

Activin A concentrations in the conditioned media were measured by an enzyme-linked immunosorbent assay (ELISA) (Knight *et al.* 1996) using a commercially available kit (MCA 1426KZZ) according to the supplier's instructions (Serotec, Oxford, Oxon, UK). The sensitivity of the assay was <100 pg/ml. Inter- and intraplate coefficients of variation were $<10\%$. The assay has no detectable cross-reaction with inhibin A, inhibin B, activin B or follistatin.

Statistics

Differences in the activin βA mRNA levels and activin A concentrations were assessed by Mann–Whitney test. The level of significance was chosen as $P<0.05$.

Results

We screened 20 pheochromocytomas and nine normal adrenal tissues for activin βA mRNA expression. Northern blots hybridized with the oligonucleotide probes revealed a predominant activin βA transcript of about 6.0 kb in size in pheochromocytomas, but the signal was very weak or not detectable in normal and the tumor-adjacent adrenal tissues (Fig. 1). The expression level of activin βA mRNA in individual pheochromocytomas varied greatly. Although there was a trend for higher levels of activin βA mRNA in the intra-adrenal benign pheochromocytomas than in malignant ones, the difference was not statistically significant ($P>0.05$). Reverse transcription-polymerase chain reaction (RT-PCR) analysis using previously validated methodology (Voutilainen *et al.* 1991) confirmed the expression of activin βA mRNA in all pheochromocytoma samples (data not shown). To clarify if the locally expressed activin could function auto- and/or paracrinely, we studied the expression of type I activin receptor genes (ActR-I and ActR-IB) in our pheochromocytoma samples. Specific transcripts of the expected sizes were detected for both ActR-I (4.0 kb) and ActR-IB (5.2 kb) receptor subtypes in the pheochromocytoma tissues (Fig. 1). Very weak signal for ActR-II mRNA was also detected in some pheochromocytomas, but ActR-IIB expression was not detectable by Northern blotting (data not shown). For comparison, we then hybridized the Northern blots with specific oligonucleotide probes for inhibin α and βB subunit mRNAs

Figure 1 Expression of mRNAs for activin β A subunit and type I activin receptors (ActR-I and ActR-IB) in benign pheochromocytomas and the tumor-adjacent adrenal tissues *in vivo*. Total RNA was extracted from the frozen tissues, and the Northern blot was prepared with 20 μ g total RNA for each lane. The filter was sequentially hybridized with 32 P-labeled activin β A subunit, ActR-I and ActR-IB oligonucleotide probes, and a 28S ribosomal RNA cDNA probe.

(Voutilainen *et al.* 1991). There was no detectable expression of inhibin α or β B subunit mRNA in the pheochromocytoma samples, although inhibin α mRNA was highly expressed in the normal and tumor-adjacent adrenal tissues (data not shown).

In primary cultures of pheochromocytoma cells, expression of activin β A mRNA was readily detectable. The accumulation of activin β A mRNA was induced two- to fivefold in repeated experiments ($P < 0.05$) by $(\text{Bu})_2\text{cAMP}$ (1 mM) after 3 days of treatment (Fig. 2). The increase of activin β A mRNA content by $(\text{Bu})_2\text{cAMP}$ was dose dependent, and this stimulation was already detectable after 24 h of treatment (data not shown). In contrast, prolonged treatment with a protein kinase C modulator, 12-O-tetradecanoyl phorbol-13-acetate (TPA; 160 nM), for 3 days had no significant effect on the basal activin β A mRNA level, but it augmented the stimulatory effect of $(\text{Bu})_2\text{cAMP}$ (Fig. 2). Treatment with a protein kinase inhibitor, staurosporine (100 nM), inhibited both the basal and $(\text{Bu})_2\text{cAMP}$ -induced expression of activin β A mRNA more than 70% (Fig. 3). The basal and $(\text{Bu})_2\text{cAMP}$ -induced expression of activin β A mRNA was also

Figure 2 The effects of $(\text{Bu})_2\text{cAMP}$ (1 mM) and TPA (160 nM) on activin β A mRNA expression in cultured pheochromocytoma cells. The cells were first cultured without stimulation for 6 days, and then treated with the agents for 3 days. The Northern blot was prepared with 10 μ g cytoplasmic RNA in each lane. The filter was hybridized with activin β A subunit and 28S RNA probes. The migration of 28S and 18S ribosomal RNAs is indicated. The experiment was repeated twice and the results were similar.

inhibited about 50% during chromaffin differentiation induced by dexamethasone (1 μ M for 3 days; Fig. 4).

To investigate if activin receptor ActR-I and ActR-IB are also regulated in cultured pheochromocytoma cells, we hybridized the Northern blots with the activin receptor probes. Although both ActR-I and ActR-IB mRNAs were detectable in the cultured cells, the tested agents mentioned above had no significant effect on the accumulation of these two type I activin receptor mRNAs (Fig. 5).

We used a specific ELISA for measuring the secretion of activin A by the cultured pheochromocytoma cells. There was no detectable activin A in the blank medium supplemented with 10% fetal calf serum. All conditioned media from cultured pheochromocytoma cells contained measurable amounts of activin A. Secretion of activin A was increased about 150% by $(\text{Bu})_2\text{cAMP}$ (1 mM) treatment for 3 days. However, dexamethasone (1 μ M) inhibited the basal and $(\text{Bu})_2\text{cAMP}$ -induced secretion of activin A from cultured pheochromocytoma cells about 45% (Fig. 6). In contrast, there was no detectable inhibin A secretion into the conditioned media measured by an inhibin A-specific ELISA (kit code, MCA 1273KZZ; Serotec).

Figure 3 The effects of (Bu)₂cAMP (1 mM) and staurosporine (ST; 100 nM) on activin β A subunit mRNA expression in cultured pheochromocytoma cells. The cells were cultured and the Northern blot was prepared as described in Fig. 2. The experiment was repeated three times with cells from different patients, and the results were comparable.

Discussion

We investigated the mRNA expression of activin subunits and activin receptors in human pheochromocytomas

Figure 4 The effects of dexamethasone (DEX; 1 μ M) and (Bu)₂cAMP (1 mM) on activin β A subunit mRNA expression in cultured pheochromocytoma cells. The cells were cultured and the Northern blot was prepared as described in Fig. 2. The experiment was repeated three times with cells from different patients, and the results were similar.

Figure 5 Expression of type I activin receptor ActR-I and ActR-IB mRNAs in cultured pheochromocytoma cells. The dispersed cells were first cultured for 7 days and then treated with (Bu)₂cAMP (1 mM), TPA (160 nM) and staurosporine (ST; 100 nM) for 3 days. The Northern blot was prepared with 10 μ g cytoplasmic RNA for each lane, and the filter was sequentially hybridized with ActR-I, ActR-IB and 28S RNA probes. The experiment was repeated three times with cells from different patients, and the results were comparable.

in vivo, and the regulation of the expression of these genes in cultured pheochromocytoma cells. Activin β A subunit gene was expressed in human pheochromocytomas. It has previously been reported that inhibin α subunit is not detectable by immunohistochemistry in most human pheochromocytomas (Chivite *et al.* 1998, McCluggage *et al.* 1998, Pelkey *et al.* 1998). Our results confirmed that inhibin α and β B subunit mRNAs are not detectable in pheochromocytomas. As both benign and malignant tumors expressed activin β A gene, this cannot be used as a discriminator between benign and malignant pheochromocytomas. We believe that the locally expressed activin β A subunits are able to assemble into activin A homodimers and function through auto- or paracrine pathways in pheochromocytoma tissues. The present study shows that activin A, but not inhibin A, is secreted by cultured pheochromocytoma cells. Since type I and II activin receptor genes were also expressed in these pheochromocytomas, all principle components of the machinery required for auto- or paracrine action of activin A are present in human pheochromocytomas. Previous reports have already demonstrated that activin A is indeed able to induce neuronal differentiation of rat pheochromocytoma PC12 cells, and to suppress cell proliferation in this cell

Figure 6 Regulation of activin A secretion by (Bu)₂cAMP (1 mM) and dexamethasone (DEX; 1 μ M) in cultured pheochromocytoma cells. The dispersed cells were first cultured without stimulation for 5–7 days, and then treated with the agents for 3 days. The conditioned media were collected and activin A concentration was measured by ELISA. Each bar represents the means $\pm$ S.E.M. of pooled data from experiments with five different pheochromocytomas with the mean of the controls adjusted to 100. ** $P < 0.001$ and * $P < 0.05$ compared with the control.

line (Paralkar *et al.* 1992, Hashimoto *et al.* 1993, Iwasaki *et al.* 1996).

The enhancer region of human activin β A gene contains two short elements with similarity to cAMP and phorbol ester response elements (Tanimoto *et al.* 1996). Expression of activin β A mRNA is induced by cAMP analogs and TPA in different cell types, including adrenal and ovarian granulosa cells (Voutilainen *et al.* 1991, Tuuri *et al.* 1996). Therefore, we wondered whether expression of activin β A mRNA in human pheochromocytoma cells is also regulated by these protein kinase modulators. In primary cultures of human pheochromocytoma cells, expression of activin β A gene and secretion of activin A was indeed increased by (Bu)₂cAMP treatment, and this stimulatory effect was augmented by combined treatment with TPA. These effects are likely to be mediated through the activation of protein kinases since the protein kinase inhibitor, staurosporine, inhibited the stimulation. Thus,

the production of activin A in pheochromocytoma cells may be associated with neuronal phenotype, since both (Bu)₂cAMP and TPA have previously been reported to induce neuronal differentiation of PC12 cells (Glowacka & Wagner 1990).

To test whether the stimulatory effects of (Bu)₂cAMP and TPA on activin A expression can be antagonized by induction of chromaffin differentiation, we used dexamethasone to treat the cells. Dexamethasone has been shown to induce chromaffin differentiation, but to inhibit neuronal differentiation in long-term cultures of human pheochromocytoma cells (Brown *et al.* 1998). In our study, dexamethasone inhibited both the constitutive and (Bu)₂cAMP-induced expression of activin β A mRNA, as well as the secretion of activin A protein. This is in agreement with previous reports showing that activin β A gene expression is inhibited by dexamethasone treatment in other cell types (Yu *et al.* 1996, Shao *et al.* 1998). The inhibitory effect of glucocorticoids *in vivo* may be the reason for the spontaneous induction of activin β A mRNA expression in cultured cells. Human pheochromocytoma cells in primary cultures develop spontaneously long neurite-like processes (Pfragner & Walser 1980, Liu *et al.* 1994), and contain increased amounts of neuropeptide Y, leading to the expression of neuronal phenotype (Tischler *et al.* 1985). These changes may be due to the relief from the inhibitory effect of the high glucocorticoid concentrations *in vivo*. Our results show that this culture-induced neuronal differentiation is also associated with increased activin β A gene expression in pheochromocytoma cells.

In conclusion, our results show that activin A and its receptors are expressed in human pheochromocytoma tissues. Production of activin A in cultured pheochromocytoma cells is associated with the neuronal differentiation status, but reduced during chromaffin differentiation. Therefore, activin A may function as a local neurotrophic factor via an auto- and/or paracrine manner in pheochromocytomas. Further studies are needed to clarify the utility of activin A expression as a marker for neuronal/chromaffin differentiation of pheochromocytomas.

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