Mitogenic Response of Human SH-SY5Y Neuroblastoma Cells to Insulin-like Growth Factor I and II Is Dependent on the Stage of Differentiation

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Abstract. Human insulin-like growth factor I and II (IGF-I and IGF-II) in concentrations of 1–30 ng/ml, were shown to stimulate ornithine decarboxylase activity and [^{3}H]thymidine incorporation in human SH-SY5Y neuroblastoma cells. Proliferation of these cells was also stimulated by IGF-I and II when added to RPMI 1640 medium, fortified with selenium, hydrocortisone, transferrin, and β-estradiol. Labeled IGF-I and II bound to SH-SY5Y cells. The cross-reaction pattern of IGF-I, IGF-II, and insulin in competing with the binding of labeled IGF-I and IGF-II, respectively, indicated that SH-SY5Y cells express both type I and type II IGF receptors. Treatment of SH-SY5Y cells for 4 d with 12-O-tetradecanoylphorbol-13-acetate (TPA), which resulted in morphological and functional differentiation and growth inhibition, abolished the mitogenic response to both IGF-I and II. Concomitantly, the binding of IGF-II disappeared almost totally, which offers a possible explanation for the reduced biological response to IGF-II after TPA treatment. In contrast, the IGF-I binding in TPA-treated cells was only reduced to ~70% of the binding to control cells. It is therefore not excluded that the IGF-I receptor could be uncoupled by TPA, with persistent binding capacity for IGF-I.

THE human adrenergic neuroblastoma cells, SH-SY5Y, differentiate when exposed to biologically active phorbolesters (22, 24, 30) or retinoic acid (23). At nanomolar concentrations, the most potent phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA),[1] induces an increase in the concentrations of noradrenalin and neuron-specific enolase (22). Morphologically, the TPA-treated cells acquire long cell processes and an increased amount of neurosecretory granula. TPA treatment also leads to increased resting membrane potential and the cells are depolarized by high concentrations of potassium (1).

TPA-treated SH-SY5Y cells are partially growth inhibited (22) and have a low ornithine decarboxylase (ODC) (EC 4.1.1.17) activity, which cannot be stimulated by a change to fresh medium (16). ODC is the rate-limiting enzyme in the biosynthesis of polymines (8, 27) and during mitogenic stimulation of cultured cells, including SH-SY5Y cells (16), the ODC activity is rapidly induced (18). By use of the irreversible ODC inhibitor, α-difluoromethylornithine, an association between inhibition of both ODC activity and cell proliferation has been found (Mattsson, M. E. K., and S. Påhlman, unpublished data). The lack of ODC response to serum in TPA-treated cells could indicate that these cells have lost the ability to respond to the growth-promoting activity of serum.

The mitogenic effect of serum in different cell culture systems has been attributed to the polypeptides in the somatomedin family and platelet-derived growth factor (7, 32). The two main forms of somatomedins, isolated from human serum, are insulin-like growth factor I (IGF-I) and insulin-like growth factor II (IGF-II) (25, 26). The polypeptide termed somatomedin C was recently shown to be identical to IGF-I (12), while the polypeptide termed somatomedin A differs from IGF-I by deamidation of glutamine in position 40 (5). An IGF-II homologue, multiplication-stimulating activity has been isolated from conditioned medium of rat BRL-cells (21).

The present study shows that both IGF-I and II promote growth of SH-SY5Y cells and that the mitogenic effect of these factors in TPA-differentiated cells is essentially lost. Furthermore, the binding sites for IGF-II, present in undifferentiated cells, disappear almost completely after TPA treatment. This cell system gives an opportunity to study the growth control in differentiating SH-SY5Y neuroblastoma cells under defined conditions.

Materials and Methods

Chemicals

Human IGF-I and IGF-II were isolated from serum as previously described (5). The proteins were labeled with ^{125}I using the lactoperoxidase method yielding

[1] *Abbreviations used in this paper:* FCS, fetal calf serum; HSA, human serum albumin; IGF, insulin-like growth factor; ODC, ornithine decarboxylase; SHTE-medium, RPMI 1640 medium supplemented with selenium (30 nM), hydrocortisone (10 nM), transferrin (100 μg/ml), and β-estradiol (10 nM); Tdr, thymidine; TPA, 12-O-tetradecanoylphorbol-13-acetate.

specific activities of 50–100 μCi/μg. TPA was obtained from PL Biochemicals (Milwaukee, WI). A stock solution was made in ethanol and in the experiments the ethanol concentration never exceeded 0.1%. This concentration had no apparent effect on growth or differentiation of SH-SY5Y cells. Human serum albumin (HSA) was from AB Kabi (Stockholm, Sweden), β-estradiol, hydrocortisone, and transferrin were from Sigma Chemical Co. (St. Louis, MO), insulin was from Novo Industri AB (Copenhagen, Denmark), and selenious acid was from Kebo (Stockholm, Sweden). Tritiated thymidine (2 Ci/mmol) was from The Radiochemical Centre (Amersham Corp., UK).

Cell Cultures

The human adrenergic neuroblastoma cell line, SH-SY5Y (2, 3), was kindly provided by Dr. June Biedler (Sloan-Kettering Institute, New York). The cells were routinely grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum (FCS), penicillin (100 IU/ml), and streptomycin (50 μg/ml) at 37°C in a 95% air, 5% CO_2–humidified incubator.

In the experiments of thymidine incorporation, 10^6 cells were seeded per 3.5 cm dish (Falcon Labwares, Grenoble, France) and cultured for 4 d in the medium described above. Before assay, the cells were washed twice and incubated for 2 d in RPMI-HSA medium (RPMI 1640 containing 5 mg HSA/ml). Then, the medium was replaced by 1 ml fresh RPMI-HSA medium containing the mitogens to be tested. At indicated times, the cells were pulsed for 1 h with 0.2 μCi [^{3}H]thymidine ([^{3}H]Tdr) per dish. Thereafter, the cells were washed twice in cold phosphate-buffered saline and precipitated with 10% cold trichloroacetic acid. The precipitated cells were washed three times with water, removed from the dish by 0.3 M NaOH and 1% SDS, and the radioactivity was counted in a scintillation counter. The number of cells per dish (~3.5 × 10^6 cells in nontreated and 1.5 × 10^6 cells in TPA-treated cultures), was determined in parallel cultures.

For determination of cell proliferation, 5 × 10^4 cells were seeded in 3.5-cm culture dishes in 2 ml Eagle's medium supplemented with 10% FCS. The cells were allowed to plate for 1 d in this medium. Different concentrations of growth factor in RPMI medium supplemented with selenium (30 nM), hydrocortisone (10 nM), transferrin (100 μg/ml), and β-estradiol (10 nM) (SHTE medium) (29), was added to the cells. SHTE medium alone could not stimulate ODC activity, [^{3}H]Tdr incorporation or proliferation of SH-SY5Y cells. After 7 d, with change to fresh medium at day 4, the cells were harvested by trypsinization and viable (>92% of the cells), Trypan blue–excluding cells were counted in a Bürker chamber.

ODC Assay

Cells (2 × 10^6/8.5-cm dish) were grown for 4 d in Eagle's medium with 10% FCS, which was replaced by fresh RPMI medium including the mitogen to be tested. Cells were harvested at indicated times and prepared for ODC assay as previously described by Mattsson et al. (16). Protein was determined according to a modified Lowry procedure (24).

IGF-I and IGF-II Binding

The binding studies were performed either at 5°C (*A*), using detached cells in suspension, or at 23°C (*B*), with cells still attached to the culture dish.

(*A*) Nontreated SH-SY5Y cells or cells treated for the indicated times with 16 nM TPA were removed from the culture dish by EDTA treatment. Cell aggregates were disrupted mechanically using a Pasteur pipette and the cells were suspended in Eagle's medium containing 5 mg HSA per ml (binding medium) to a final concentration of 10^6 cells/100 μl medium. Labeled IGF-I and IGF-II and various amounts of cold growth factors were incubated with the cells for 6 h at 5°C (conditions for optimal IGF binding at 5°C [data not shown]). 100-μl aliquots of the incubation mixtures were layered over 1 ml of a mixture of dibutylphthalate and dinonylphthalate (at a 2:3 ratio) in Eppendorf tubes. Cell-bound IGF was separated from free IGF by spinning down the cells through the oil layer, at 13,000 rpm for 2 min in an Eppendorf centrifuge. The tips of the tubes were cut off and counted in a gammacounter.

(*B*) Nontreated cells were grown to confluency (~1.5 × 10^6 cells/well) in Linbro plates (Flow Laboratories, Inc., McLean, VA) with 24 wells. The cells were washed three times with binding medium and preincubated in 0.5 ml of this medium for 1 h. Labeled IGF-I or IGF-II were added to the cultures together with different amounts of cold growth factors. After an incubation period of 1 h at 23°C, the cells were washed five times in ice-cold binding medium before lysis in 0.3 M NaOH and 1% SDS. The number of cells per well was determined in parallel cultures. In the case of TPA-treated cells, these cultures were not confluent when assayed for binding of IGF.

Results

Activation of ODC and Thymidine Incorporation by IGF-I and II in SH-SY5Y Cells before and after TPA Treatment

The ODC activity in SH-SY5Y cells, grown for 4 d in presence of 10% FCS, was low. Change to fresh medium containing FCS, resulted in an increased ODC activity with maximal response after 6 h. Under the same initial conditions, IGF-II (30 ng/ml) in RPMI medium induced ODC activity of similar duration but of higher magnitude than the ODC activity induced by 10% FCS (Fig. 1). The kinetics of FCS-stimulated [^{3}H]Tdr incorporation in SH-SY5Y cells is shown in Fig. 2. An increase in incorporated radioactivity could be detected after 13 h and the maximal incorporation rate was obtained between 19 and 25 h after the addition of FCS. The control medium, RPMI-HSA, did not cause any obvious change in

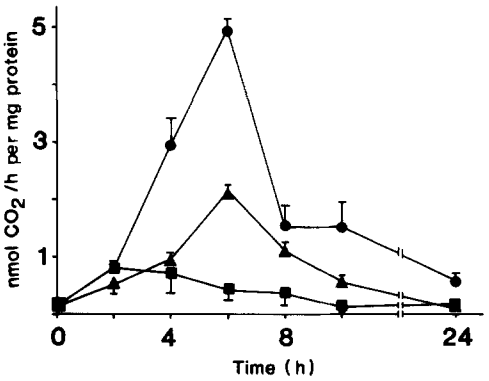

Figure 1. ODC activity in SH-SY5Y cells stimulated with FCS or IGF II. Cells were grown for 4 d in medium supplemented with 10% FCS. After a change to fresh RPMI 1640 medium containing 10% FCS (▲), 30 ng IGF-II/ml (●), or without any addition (■), the cells were harvested at the indicated times for determination of ODC activity. Each point is the mean of triplicate samples ±SD.

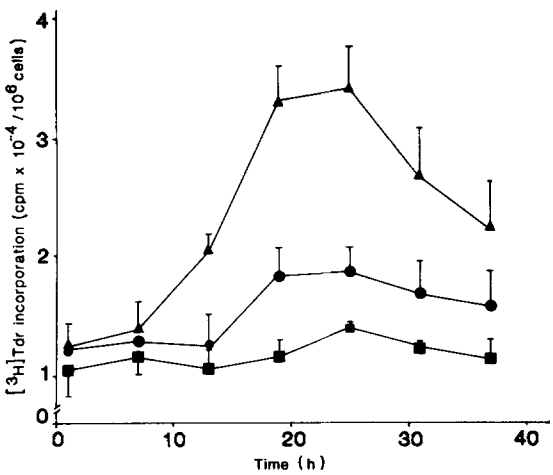

Figure 2. ^{3}H-Tdr incorporation in SH-SY5Y cells stimulated with FCS or IGF-II. Cells were grown for 4 d and then serum depleted for 2 d before change to RPMI-HSA medium (■), supplemented with 10% FCS (▲) or 30 ng IGF-II/ml (●). At the indicated times, the cells were pulsed for 1 h with ^{3}H-Tdr (0.2 μCi/ml) and processed as described in Materials and Methods. The number of cells per dish was determined in parallel cultures. Each point is the mean of triplicate cultures ±SD.

[^{3}H]Tdr incorporation. Addition of IGF-II (30 ng/ml) to the medium, caused an increase of [^{3}H]Tdr incorporation during the same time period but of lower magnitude than that found in FCS-stimulated cells (Fig. 2). IGF-I could also induce the activation of ODC and [^{3}H]Tdr incorporation with a similar kinetics as for the stimulation by IGF-II (not shown).

The activation of ODC by IGF-I or IGF-II was concentration dependent with a maximal response at concentrations above 10 ng/ml (Fig. 3). The activity induced by IGF-I was similar to that induced by IGF-II. Similarly, the stimulated [^{3}H]Tdr incorporation was concentration dependent with maximal response at concentrations above 10 ng/ml of IGF-I or IGF-II (Fig. 3).

After 4 d of treatment with 16 nM TPA, when the SH-SY5Y cells were morphologically and functionally differentiated, IGF-I and II in RPMI medium caused only a slight induction of ODC activity. Similarly, IGF-I and II had no or very little stimulatory effect on [^{3}H]Tdr incorporation of cells treated for 4 d with TPA (Fig. 3).

Proliferation of SH-SY5Y Cells in a Defined Medium Containing IGF-II

To investigate the mitogenicity of IGF polypeptides, SH-SY5Y cells were exposed to different concentrations of IGF-II in RPMI or RPMI-HSA medium, but after 5–6 d in culture, the cells began to detach from the culture dish. However, the SH-SY5Y cells could be grown for at least 2 mo in SHTE medium together with a high concentration of insulin (5 μg/ml). IGF-II in combination with SHTE medium supported proliferation of SH-SY5Y cells. The proliferation rate was dose dependent, with maximal growth at concentration of 10–30 ng IGF-II/ml (Fig. 4). The dose dependency of proliferation was similar to the activation of ODC and thymidine incorporation. However, the proliferation rate was lower than in cells grown in Eagle's medium containing 10% FCS (not shown). Also 10–30 ng IGF-I/ml in SHTE medium could support proliferation of the SH-SY5Y cells (not shown).

The decrease in IGF mediated inducibility of the ODC activity and the [^{3}H]Tdr incorporation after TPA treatment indicated that these cells no longer could respond to IGF-I or II with proliferation. Experiments to verify this failed, because the TPA-treated cells adhered poorly in SHTE–IGF-I or SHTE–IGF-II medium and it was not possible to follow the proliferation for a longer period.

IGF-I and IGF-II Binding to SH-SY5Y Cells before and after TPA Treatment

Cells, incubated with ^{125}I–IGF-I or II at 5°C or 23°C displayed maximal binding after 6 h and 1 h, respectively (not shown). Both labeled IGF-I and II bound specifically to the SH-SY5Y cells at 5°C (Fig. 5). IGF-II was slightly less potent than IGF-I in displacing labeled IGF-I, and insulin cross-reacted with IGF-I for its receptor. IGF-II was considerably more potent than IGF-I in displacing labeled IGF-II (Fig. 5 *B*). Physiological concentrations of insulin did not cross-react with IGF-II while a displacement could be seen at pharmacological concentrations (100–3,000 ng/ml).

The specific binding of IGF-II to SH-SY5Y cells was virtually lost after TPA treatment for 4 d (Fig. 6). The changes in the kinetics of the binding of IGF-I and II to TPA-treated SH-SY5Y cells is shown in Fig. 7. After 30 min of TPA treatment, a slight decrease in IGF-II binding was seen and prolonged TPA treatment resulted in a further reduction of the IGF-II binding, with 15 and 5% left of the initial binding, after 24 and 96 h of treatment, respectively. In contrast, specific binding of IGF-I was reduced to ~70% within 30 min

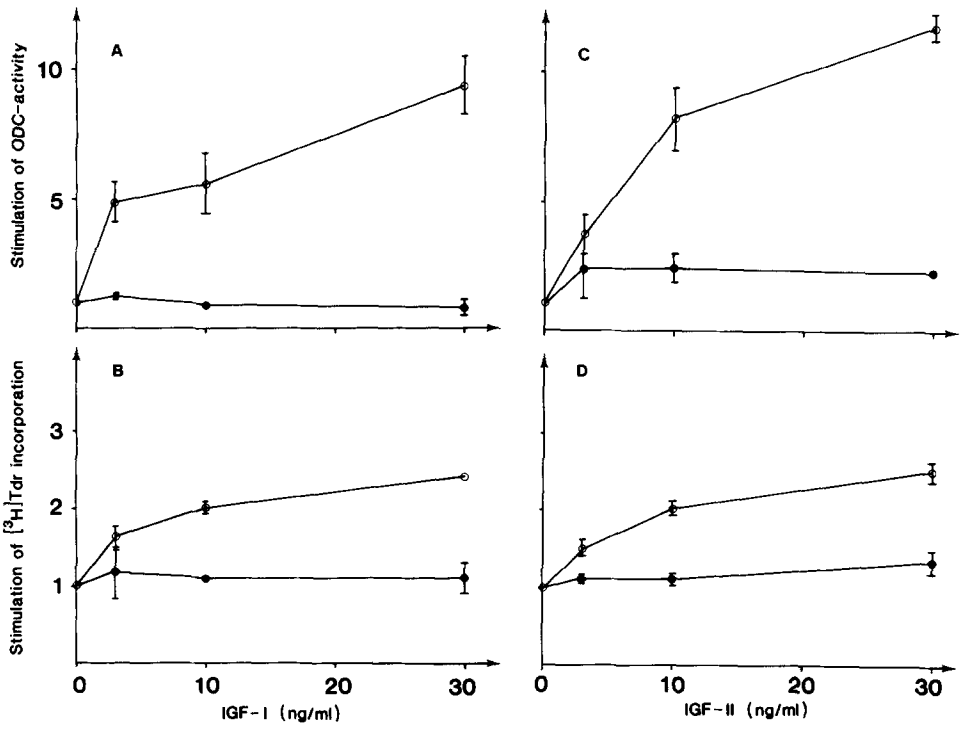

Figure 3. Stimulation of ODC activity and ^{3}H-Tdr incorporation by IGF-I and IGF-II in non-treated and TPA-treated SH-SY5Y cells. The IGF-induced response has been compared to the stimulation obtained when no IGF was present. Cells were grown for 4 d with (○) or without (●) 16 nM TPA present in FCS-supplemented medium. (*A*) Fresh RPMI medium with IGF-I in concentrations between 3–30 ng/ml was added and the cells were harvested after 5 h for determination of ODC activity. Each point is the mean of triplicate samples ±SD. (*B*) The cells were serum depleted for 2 d and then stimulated with RPMI-HSA medium with different IGF-I concentrations. After 23 h, the cells were pulsed for 1 h with ^{3}H-Tdr and processed as described in Materials and Methods. Each point is the mean of duplicate cultures. (*C*) Activation of ODC by IGF-II. Conditions as in *A*. (*D*) ^{3}H-Tdr incorporation induced by IGF-II. Conditions as in *B*.

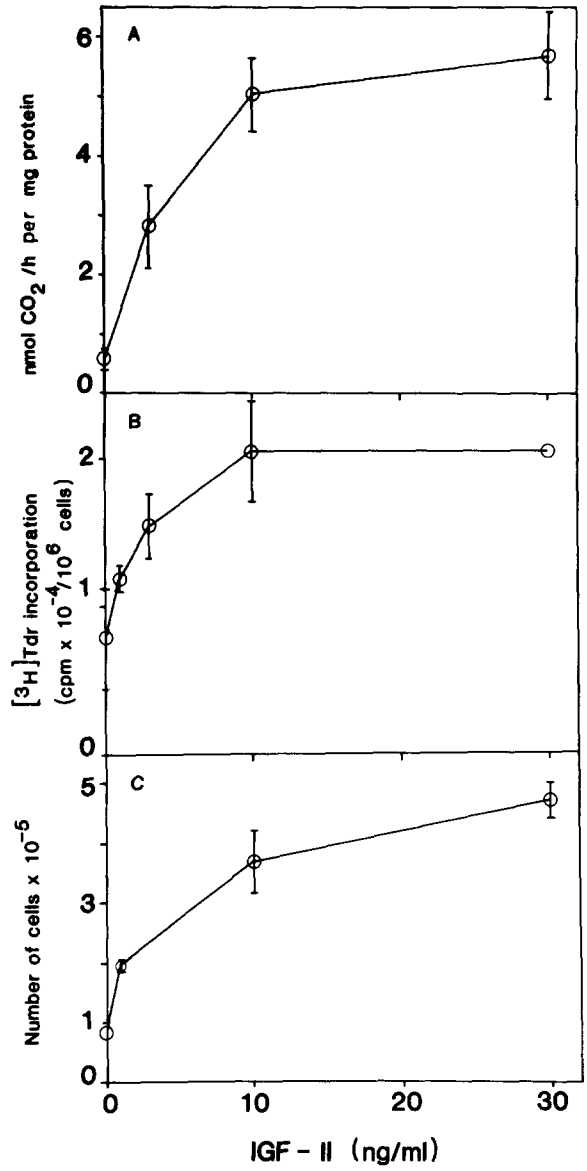

Figure 4. (*A*) ODC activity, (*B*) ^{3}H-Tdr incorporation, and (*C*) proliferation of SH-SY5Y cells stimulated with 1–30 ng/ml of IGF-II. (*A* and *B*) Conditions as in Fig. 3*A* and *B*. (*C*) Cells were plated in FCS-supplemented medium. After 1 d, it was changed to SHTE medium with different concentrations of IGF-II. The cells in duplicate cultures were counted after 7 d of culture.

after TPA treatment. Prolonged treatment did not result in a further reduction of IGF-I binding.

Discussion

The growth-promoting effect of physiological concentrations of polypeptide hormones is mediated through specific membrane-bound receptors. Changes in the expression and/or structure of these receptors could be a mechanism for the control of growth of SH-SY5Y cells. These cells proliferate in FCS-supplemented medium and decrease their proliferation rate markedly during TPA-induced differentiation (22, 24). In such cells, ODC activity was no longer inducible by FCS (16). In the present study we show that SH-SY5Y cells have binding sites for IGF-I and II; growth factors present in serum.

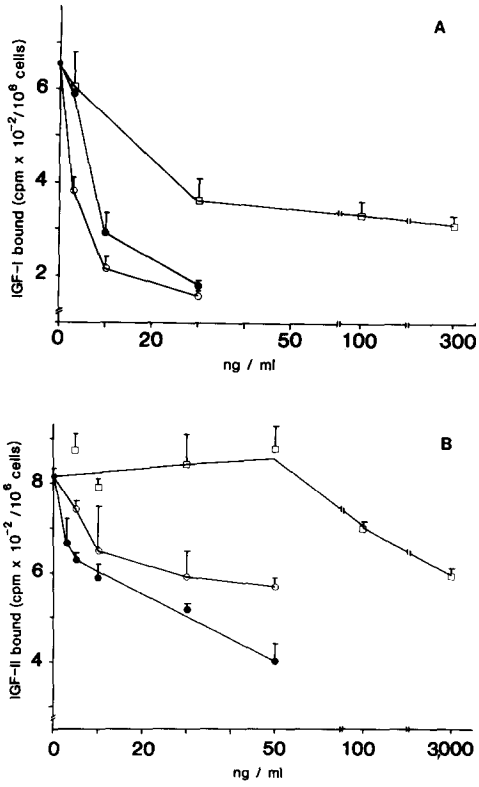

Figure 5. Binding of ^{125}I-IGF-I and ^{125}I-IGF-II to SH-SY5Y cells. Cells were incubated in binding medium at 5°C for 6 h with labeled IGF (8,000 cpm/10^6 cells) and different amounts of unlabeled IGF-I (○), IGF-II (●), or insulin (□). The cell-bound radioactivity in 10^6 cells was determined after the cells had been separated from free tracer as described in Materials and Methods. (*A*) Bound ^{125}I-IGF-I. (*B*) Bound ^{125}I-IGF-II. Each point is the mean of triplicate determinations ±SD.

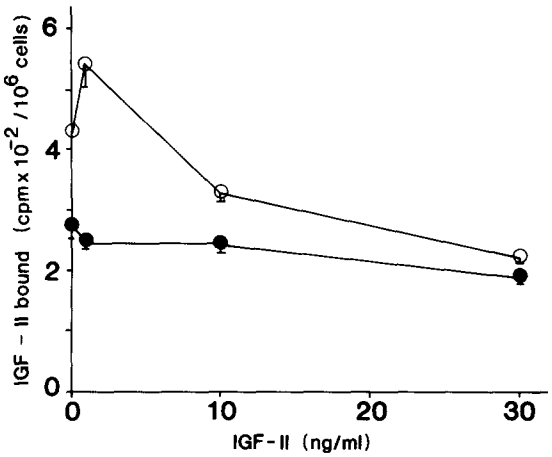

Figure 6. Binding of ^{125}I-IGF-II to non-treated and TPA-treated SH-SY5Y cells. The cells were grown for 4 d with (●) or without (○) 16 nM TPA. The cultures were incubated with ^{125}I-IGF-II (25,000 cpm/well) and different amounts of unlabeled IGF II at 23°C for 1 h. The cell-bound radioactivity was measured in duplicates after the cells had been washed and solubilized. The number of cells was counted in parallel cultures.

Furthermore, pure human IGF-I and II stimulated the ODC activity and thymidine incorporation in SH-SY5Y cells, before but not after TPA treatment. The defined SHTE medium

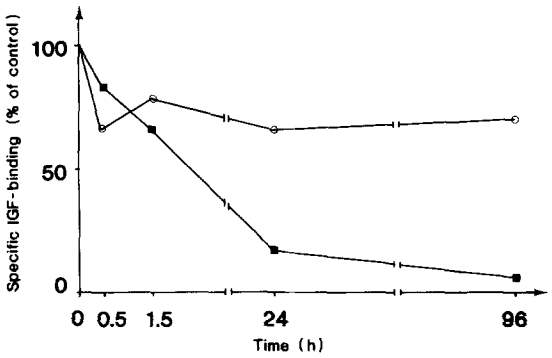

Figure 7. Specific ^{125}I-IGF-I and II binding to SH-SY5Y cells treated by TPA for various time periods. After the treatment with 16 nM TPA, the cells were harvested and split into two samples for incubation at 5°C with IGF-I and IGF-II as detailed in Materials and Methods and Fig. 5. The binding of IGF-I (○) and IGF-II (■) was expressed as % of the binding to non-treated SH-SY5Y cells.

supplemented with these factors could support the proliferation of SH-SY5Y cells.

Although IGF-I or IGF-II, in SHTE medium, promoted proliferation of SH-SY5Y cells over several cell divisions, this medium did not provide optimal growth conditions. Apart from a lower proliferation rate in SHTE–IGF-II medium, as compared with medium containing 10% FCS, the cells tended to detach from the culture dish when grown for more than a week. Thus, serum is superior to SHTE–IGF-II medium in stimulating DNA synthesis and maintaining growth. Serum contains other growth factors such as platelet-derived growth factor. However, the SH-SY5Y cells lack binding sites for this factor (9) and the higher potency of serum has to be attributed to unknown serum growth and/or maintenance factors.

The low concentrations (10–30 ng/ml) of IGF-I and II required for maximal stimulation of ODC activity and thymidine incorporation could suggest that these growth factors act through somatomedin/IGF receptors. Two types of somatomedin/IGF receptors have been characterized. The type I receptor prefers IGF-I over IGF-II and recognizes insulin, whereas the type II receptor prefers IGF-II over IGF-I and does not recognize insulin (21). The cross-reaction pattern of these factors indicates that the SH-SY5Y cells express both type I and type II receptors.

TPA treatment markedly decreased the IGF-I and II–induced stimulation of ODC activity and DNA synthesis despite remaining binding of IGF-I. The reduced binding of IGF-II to TPA-treated cells offers a possible explanation for the absence of a mitogenic response. The remaining IGF-I binding to TPA-treated cells could indicate that the biological response to IGF-I is mediated via the type II receptor. However, it could also be explained by a TPA-induced uncoupling of type I receptors. Such a TPA effect has been described for the α_1-adrenergic receptor in hamster smooth muscle cells, where TPA treatment results in a receptor uncoupling from inositol phospholipid metabolism, despite an unaltered agonist binding (14). Therefore, the present results cannot elucidate whether the mitogenic effects of IGF-I and II in non-treated cells are mediated through the IGF-I and/or IGF-II receptor.

Biologically active phorbol esters and other tumor promoters, like mezerein, saccharin, and cyclamate, have previously been shown to diminish the binding of several other growth factors such as epidermal growth factor (4, 13, 28), insulin (6, 31), nerve growth factor (10), and transferrin (17). The decreased binding has been attributed to either reduced receptor affinity (4, 6, 10, 28) or decreased number of receptors (13, 17).

At present, no general mechanism can explain the effect of phorbol esters on expression of growth factor receptors, but TPA has been shown to enhance the phosphorylation of insulin, IGF-I, epidermal growth factor, and transferrin receptors, respectively (11, 17, 19). Furthermore, in a phosphorylation-dependent process, TPA causes a reversible internalization of the transferrin receptor complex (17). Whether the decreased IGF-II binding observed in TPA-treated SH-SY5Y cells is a direct TPA effect or is due to the TPA-induced differentiation is not clear. The fact that most of the IGF-I and IGF-II binding is still present after 1.5 h of TPA treatment could indicate that mechanisms other than those operating in the internalization of the transferrin receptor (e.g., phosphorylation of the receptor by protein kinase C) (17) are acting in TPA-treated SH-SY5Y cells. There are reports of other types of cells that lose their binding sites for growth factors during differentiation (15, 20).

In conclusion, IGF-I and II are shown to be growth factors for SH-SY5Y cells although they cannot completely replace serum. The decrease in IGF-II binding after TPA treatment offers a possible mechanism for growth control in differentiating neuroblastoma cells.

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