Thyroid hormone induces cerebellar astrocytes and C6 glioma cells to secrete mitogenic growth factors

A. G. TREN'TIN,1 M. ALVAREZ-SILVA,2 AND V. MOURA NETO2
1Deamento de Biologia Celular, Embriologia e Genética, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, 88040-900 Florianópolis, Santa Catarina; and
2Deamento de Anatomia, Instituto de Ciências Biomédicas, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, 21949–900 Rio de Janeiro, Rio de Janeiro, Brazil

Received 28 November 2000; accepted in final form 25 June 2001

Thyroid hormone induces cerebellar astrocytes and C6 glioma cells to secrete mitogenic growth factors. Am J Physiol Endocrinol Metab 281: E1088–E1094, 2001.—In this study, the effect of thyroid hormone (triiodothyronine, T3) on the secretion of mitogenic growth factors in astrocytes and C6 glioma cells was examined. The proliferative activity of T3 could be due, at least in part, to the astrocyte secretion of acidic and basic fibroblast growth factor (aFGF and bFGF), tumor necrosis factor-β, and transforming growth factor-β. In contrast, the conditioned medium (CM) of T3-treated C6 cells was mitogenic to this cell line only after hyaluronidase digestion, suggesting the impairment of growth factor mitogenic activity by hyaluronic acid. Furthermore, the presence of bFGF was significantly greater in the CM of both T3-treated astrocytes and T3-treated C6 cells than in the corresponding control CM. These data show that T3 induces cerebellar astrocytes to secrete mitogenic growth factors, predominantly bFGF, that could influence astrocyte and neuronal proliferation via autocrine and paracrine pathways.

MATERIALS AND METHODS

Materials

Cell cultures. Primary cultures of astrocytes were prepared from cerebella obtained from 2- to 3-day-old Wistar rats as described (34–37). Cells were grown to semiconfluence (7–8 days) in the presence of Dulbecco’s modified Eagle’s medium and nutrient mixture F-12 (DMEM-F-12, Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS; Fazenda Pigue, Rio de Janeiro, Brazil). The cultures were incubated at 37°C in a humidified 5% CO2-95% air atmosphere.

The C6 cell line was grown in the presence of DMEM (Sigma) supplemented with 10% FCS. The cultures were incubated at 37°C in a humidified 5% CO2-95% air atmosphere. Cells were harvested with trypsin (0.125%, Sigma) when they reached confluence.

Cell treatment and conditioned medium preparation. After confluence, astrocyte or C6 monolayers were washed three times with serum-free DMEM-F-12 and treated with 50 nM 3,3',5-triiodo-L-thyronine (T3) in DMEM-F-12 for 3 days, with medium renewed every day except after the 3rd day. The hormone solution was dissolved in DMEM-F-12 without FCS.
Control cultures were maintained in DMEM-F-12 without FCS. Control and hormone-treated cultures were then maintained for 2 days without medium changes. The conditioned medium (CM) obtained from control (CCM) and T3-treated cells (T3CM) was collected on the 2nd day after the end of T3 treatment, filtered in nitrocellulose membrane (0.22 μm, Millipore), and stored at −20°C for later use.

**Antibodies.** Antibody anti-T3 (1:6,000, Sigma) was used in the evaluation of T3 content in T3CM by RIA and biological activity analysis.

For CM neutralization assays, neutralizing antibodies to growth factors were added to the CMs: rabbit anti-fibroblast growth factor-acidic (anti-aFGF, 1:200; Sigma); rabbit anti-fibroblast growth factor-basic (anti-bFGF, 1:450; Sigma); rabbit anti-tumor necrosis factor-β (anti-TNF-β, 1:3,500; Sigma); mouse anti-transforming growth factor-β (anti-TGF-β, 1:1,000; a gift from Dr. R. Borovevic, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil); mouse anti-interleukin-3 (anti-IL-3, 1:5,000; Sigma), and mouse anti-granulocyte macrophage colony-stimulating factor (anti-GM-CSF, 1:800; Sigma). Dilution curves were done to verify the ideal dilution of antibodies to neutralize the growth factors’ mitogenic activity (Table 1). We tested by ELISA the activity of the antibodies in the presence of recombinant growth factors, and any cross-reactivity was observed (data not shown).

**Quantitative Analysis of Astrocyte and C6 Number**

C6 monolayers were cultured in 96-well culture plates and treated with DMEM-F-12 or 50 nM T3 as described. After 4 days, the monolayers were washed with PBS, trypsinized (0.125%), and counted in a hemocytometer under a Zeiss optic microscope.

**Table 1. Dilution curve of growth factors neutralizing antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>% Inhibition of Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-aFGF</td>
<td>1:100</td>
<td>18 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>1:150</td>
<td>16 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>1:200</td>
<td>16 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>1:400</td>
<td>10 ± 0.04</td>
</tr>
<tr>
<td>Anti-bFGF</td>
<td>1:230</td>
<td>15 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>1:345</td>
<td>32 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>1:450</td>
<td>46 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>1:920</td>
<td>7 ± 0.04</td>
</tr>
<tr>
<td>Anti-TGF-β</td>
<td>1:500</td>
<td>12 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>1:750</td>
<td>15 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>1:1,000</td>
<td>17 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>1:2,000</td>
<td>10 ± 0.014</td>
</tr>
<tr>
<td>Anti-IL-3</td>
<td>1:2,500</td>
<td>23 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>1:3,750</td>
<td>24 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>1:5,000</td>
<td>28 ± 0.007</td>
</tr>
<tr>
<td></td>
<td>1:8,000</td>
<td>21 ± 0.002</td>
</tr>
<tr>
<td>Anti-TNF-β</td>
<td>1:1,750</td>
<td>3 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>1:3,500</td>
<td>5 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>1:5,000</td>
<td>1 ± 0.03</td>
</tr>
<tr>
<td>Anti-GM-CSF</td>
<td>1:400</td>
<td>1 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>1:800</td>
<td>2 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>1:1,600</td>
<td>0 ± 0.003</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SD of 3 independent experiments performed in quadruplicate. aFGF, acidic fibroblast growth factor; bFGF, basic FGF; TGF-β, transforming growth factor-β; TNF-β, tumor necrosis factor-β; GM-CSF, granulocyte macrophage colony-stimulating factor. C6 cells were cultured with astrocyte thyroid hormone (T3)-treated conditioned medium (T3CM), and the cell proliferation was measured by MTT assay.

Astrocytes were cultured in 24-well culture plates and treated with DMEM-F-12 or 50 nM T3 as described. After 4 days, the monolayers were fixed and stained with 1% trypan blue solution in PBS for 5 min, and the cell number was quantified. At least eight fields were counted per well.

**Cell Proliferation Monitoring**

**MTT assay.** The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay method has been extensively used to determine glial cell proliferation (36, 37) and as an in vitro test system for T3 action (14). C6 cells were plated (10⁴ cells/well) in 96-well culture plates and cultured for 4 days. Viable cells were quantified by MTT colorimetric assay for mitochondrial dehydrogenase, as previously described (22). Results were expressed in absorbance at 550 nm.

Primary cerebellar astrocyte cultures were prepared in 96-well culture plates as described and were maintained in DMEM-F-12 enriched with 10% FCS for 4 days until the cells reached semiconfluence. After that, the astrocyte cultures were extensively washed with PBS and submitted to the experimental conditions (described in figure legends). After 4 days, viable cells were quantified by MTT colorimetric assay as described.

**Bromodeoxyuridil incorporation and detection.** Alternatively, the cell proliferation was analyzed by 5-bromo-2'-deoxyuridine (BrdU) incorporation. Astrocyte and C6 cultures were incubated for 24 h in the presence of 1 μg/ml of BrdU (Sigma). The cells were fixed with 4% paraformaldehyde for 20 min. Cultures were washed twice with distilled water and then incubated twice in 2 N HCl at 50°C for 15 min. After that, cultures were washed twice with 0.1 M borate buffer for 10 min at room temperature. The cells were then immunoreacted with anti-BrdU antibody (as described in Sytox green nucleic acid stain) and visualized under an epifluorescent Olympus microscope (Olympus, Tokyo, Japan). The mitotic index was measured by counting the percentage of labeled cells in at least eight different fields per coverslip.

**Immunofluorescence.** The immunofluorescence was performed as described previously (36, 37). After the BrdU procedure, the monolayers were washed with PBS and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. Cells were incubated with 10% normal goat serum (Sigma) in PBS for 1 h and subsequently with rat anti-BrdU antibody (1:500; Accurate Chemical and Scientific, Westbury, NY). The cells were then washed in PBS and incubated with goat anti-rat antibody conjugated with biotin (1:400; Vector Laboratories, Burlingame, CA). The secondary antibody was revealed by incubation with Texas red-streptavidin conjugate according to the manufacturer’s instructions (Vector Laboratories). In all cases, no reactivity was observed when the primary antibody was absent. Cell nuclei were stained with dianaminophenylindole (DAPI, Sigma). The cultures were mounted on N-propyl-gallate-glycerol and examined under an epifluorescent Olympus microscope.

**Sytox green nucleic acid stain.** The astrocyte and C6 monolayers were washed with HEPES buffer (5 mM HEPES; 125 mM NaCl; 5.5 mM KCl; 1.0 mM MgCl₂·6H₂O, 1.8 mM CaCl₂·2H₂O), pH 7.4, and incubated during 10 min with Sytox green nucleic acid stain according to the manufacturer’s instructions (Molecular Probes, Eugene, OR). Cells were then washed with PBS and fixed with 4% paraformaldehyde for 20 min. Cell nuclei were stained with DAPI. The cultures were mounted on N-propyl-gallate-glycerol and examined under an epifluorescent Olympus microscope. Eight fields were counted for each well of experiment.
ELISA. The ELISA was performed as previously described (1). The proteins of CCM and T3CM were lyophilized and quantified by Bradford’s method (3). MaxiSorb immunoplates were preincubated overnight at 4°C with 2 μg/well of CCM or T3CM protein or with 0.2 to 100 ng/well of recombinant bFGF (rhFGF, Gibco-BRL, Grand Island, NY) dissolved in PBS. They were washed and subsequently incubated for 1 h at 37°C with PBS containing 0.1% Tween 20 (Sigma) and 2% bovine serum albumin (Sigma), washed again, and reacted with polyclonal antibody against bFGF (1:2,000, Sigma). The immune reaction was quantified by peroxidase-labeled anti-IgG rabbit antibody (Sigma).

C6 CM digestion. C6 CCM or T3CM was prepared as described in Cell treatment and conditioned medium preparation. The CMs were digested with hyaluronidase (100 μU/ml, Sigma) or with hyaluronidase (100 μU/ml) plus heparitinase III (10 μU/ml, Sigma) for 15 h at 37°C in a humidified 5% CO2-95% air atmosphere as described (2). The CMs were then sterilized by filtration in nitrocellulose membrane (0.22 μm, Millipore) and were either used immediately or stored at −20°C for later use.

Statistical Analysis

Differences between the groups were evaluated by ANOVA and Student’s t-test, and the differences were considered significant at P < 0.05.

RESULTS

T3 Stimulates Cerebellar Astrocyte and C6 Glioma Cell Proliferation

We further investigated the effects of T3 and CM from T3-treated cerebellar astrocyte (T3CM) treatments on proliferation of cerebellar astrocytes and C6 glioma cells. We verified that both T3 and T3CM induced significant astrocyte proliferation by MTT assay or direct cell counting (Table 2). In addition, >95% of the cells were GFAP positive before and after both treatments, attesting to their astrocyte phenotype (36, 37). These results are in agreement with our previous data (36, 37) and suggest that the secretion of mitogenic growth factors can promote the T3 effects on astrocyte proliferation. In our culture conditions, these proliferative effects were not directly mediated by T3, since no residual hormone was detected (by RIA) in the CM of hormone-treated cultures (37). Alternatively, the anti-T3 antibody did not alter the mitogenic effect of T3CM (data not shown). We also verified that T3 induced significant C6 cell proliferation, with the maximum effect obtained at 50 nM (Table 2).

To investigate whether astrocyte and C6 population augmentation was due to increased proliferation or survival, we tested the BrdU incorporation. As shown in Table 2, the incorporation of BrdU was at least four times higher in T3-treated and T3CM-treated astrocytes than in control cells, confirming that T3 plus CM stimulates cerebellar astrocyte proliferation instead of cell survival. In addition, we verified that T3-treated C6 cells incorporated 30% more BrdU than the controls. However, T3CM from C6 cells did not promote a significant increase in C6 proliferation (Table 2).

To analyze the proportion of cell mortality in our culture conditions, we performed incubations with Sytox green. As shown in Table 2, the proportion of Sytox green-positive astrocytes and C6 glioma cells was very low in all treatments, indicating that the cell mortality was insignificant in our experiments. In addition, we could observe an increase in DAPI-positive cells in both T3 (61%) and T3CM-treated (69%) astrocytes and in T3-treated (50%) C6 cells when compared with controls (data not shown).

Cerebellar Astrocyte CM Induces Astrocyte Proliferation

To analyze the growth factors involved in astrocyte proliferation, some commercial neutralizing antibodies against growth factors were added to the CMs. We verified that the addition of anti-bFGF to the T3CM promoted the more significant inhibition in astrocyte proliferation (40%); however, anti-aFGF (23%) and anti-TNF-β (29%) also presented significant inhibitory effects compared with the CCM (Fig. 1A). To test the additive effect on these growth factors, anti-aFGF, anti-bFGF, and anti-TNF-β were added together. As a result we obtained 65% inhibition in astrocyte proliferation (data not shown). We also observed a basal astrocyte production of TGF-β, because the addition of the neutralizing antibody partially inhibited the mitogenic effect of both CCM and T3CM when compared with the corresponding CM without antibody (Fig. 1A).

Table 2. T3 Effects on proliferation of cerebellar astrocyte and C6 cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Treatment</th>
<th>MTT, 550 nm</th>
<th>×10⁶ cell/ml</th>
<th>BrdU-Positive Cells, %</th>
<th>Sytox Green-Positive Cells, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrocytes</td>
<td>Control</td>
<td>0.065 ± 0.005</td>
<td>19 ± 2</td>
<td>6 ± 1</td>
<td>4 ± 0.03</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>T3</td>
<td>0.14 ± 0.007†</td>
<td>34 ± 3†</td>
<td>27 ± 1.4†</td>
<td>3 ± 0.05</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>T3CM</td>
<td>0.138 ± 0.006†</td>
<td>ND</td>
<td>28.5 ± 2.5†</td>
<td>3 ± 0.06</td>
</tr>
<tr>
<td>C6 cells</td>
<td>Control</td>
<td>0.091 ± 0.004</td>
<td>6.9 ± 0.6</td>
<td>25 ± 0.5</td>
<td>1.4 ± 0.002</td>
</tr>
<tr>
<td>C6 cells</td>
<td>T3</td>
<td>0.173 ± 0.007†</td>
<td>14.1 ± 0.2†</td>
<td>55 ± 3†</td>
<td>0.2 ± 0.001*</td>
</tr>
<tr>
<td>C6 cells</td>
<td>T3CM</td>
<td>0.069 ± 0.003</td>
<td>ND</td>
<td>28 ± 2</td>
<td>0.3 ± 0.001*</td>
</tr>
</tbody>
</table>

Cells were treated with DMEM-F-12 (Control), T3 solution (50 nM), or CM, as described in MATERIALS AND METHODS. Cell proliferation was measured by MTT assay, quantitative analysis, and bromodeoxyuridil incorporation. In addition, cell mortality was measured by Sytox green nucleic acid stain incorporation. Results are means of 5 independent experiments performed in triplicate or quadruplicate (MTT). ND, not determined. *P < 0.001, †P < 0.0001 vs. control by one-way ANOVA with Dunnett’s post hoc test.
To verify whether the soluble factors secreted by cerebellar astrocytes after T3 stimulation are also mitogenic to C6 cells, proliferating experiments with the use of T3CM from cerebellar astrocytes were performed.

As shown in Fig. 2, astrocyte T3CM induced a significant dose-dependent increase in C6 cell proliferation. The proportion of 50% of astrocyte T3CM was the initial CM concentration necessary to promote a significant increase in C6 proliferation compared with astrocyte CCM. This result indicated that the growth factors secreted by cerebellar astrocyte after thyroid hormone stimulation were also mitogenic to C6 cells.

To analyze the mitogenic growth factor(s) involved in C6 proliferation, CCM and T3CM from astrocytes were incubated with neutralizing antibodies to known growth factors (Fig. 1B). We observed significant inhibition in C6 cell proliferation after the addition of anti-bFGF (35%) or anti-IL-3 (16.5%) to the astrocyte T3CM compared with CCM (Fig. 1B). Interestingly, anti-aFGF and anti-TGF-β significantly inhibited the mitogenic effect of only astrocyte T3CM compared with the same CM without antibody but not compared with the corresponding CCM, suggesting a basal astrocyte secretion of these growth factors (Fig. 1B). In addition, C6 cultures treated with astrocyte CCM presented a significant inhibition of proliferation after the addition of neutralizing anti-bFGF compared with the same CM without antibody, also suggesting a basal concentration of bFGF.

C6 Cell CM Effects on C6 Proliferation

The preceding results in this study suggest that the T3 and astrocyte T3CM mitogenic action is similar in both C6 cells and astrocytes. However, T3CM from C6 cells was not effective in promoting C6 cell proliferation (Table 2).

Hyaluronic acid is a negatively charged, high-molecular-weight polysaccharide that forms strikingly viscous solutions (19) and is overexpressed in tumor cells (32). In our culture conditions, we observed that the C6 cell CM effects on C6 proliferation were pronounced.

Cerebellar Astrocyte CM Induces C6 Cell Proliferation

To verify whether the soluble factors secreted by cerebellar astrocytes after T3 stimulation are also mitogenic to C6 cells, proliferating experiments with the use of T3CM from cerebellar astrocytes were performed.
CM was very viscous. We hypothesized that C6 cells secreted a very large amount of hyaluronic acid, which accumulated in the culture dish, interfering with growth factor activity. To test this hypothesis, we digested C6 CM with hyaluronidase or hyaluronidase plus heparitinase III and observed the recovery of mitogenic activity. As shown in Fig. 3, digested or nondigested C6 CCM had no effect on C6 proliferation. As expected, nondigested C6 T3CM did not induce C6 cell proliferation; however, a significant increase in C6 proliferation was observed when T3CM from C6 cells was digested by hyaluronidase.

In addition, anti-bFGF was added to the T3CM from C6 cells previously digested by hyaluronidase, and the proliferative activity was partially abolished (Fig. 3). Heparitinase III digestion also impaired the recovery of the proliferative effect of the C6 T3CM promoted by hyaluronidase (Fig. 3).

Heparin and C6 Proliferation

It has been demonstrated that heparin enhances the activity of the FGFs (31) and that its excess reduces the growth factor action (18). To verify whether these effects could be observed in our experimental model, a heparin concentration curve was performed with C6 cells (Fig. 4). The addition of low concentrations of heparin (0.1, 0.5, 1, or 10 ng/ml) to the 50 nM T3 solution stimulated C6 proliferation, and as expected, the higher concentrations (50 or 70 ng/ml) inhibited it.

bFGF Quantification

The aforementioned experiments with growth factors neutralizing antibodies suggest that the bFGF may be the main factor present in the CM from both astrocytes and C6 cells. To quantify this growth factor, we performed ELISA assays (Fig. 5). We verified a significant increase in bFGF production in T3CM of both astrocytes (eightfold) and C6 cells (twofold) compared with the corresponding CCM.

**DISCUSSION**

We had previously shown that T3 induces the synthesis and secretion of growth factors by cerebellar astrocytes that mediate the T3 proliferative effect (21, 36, 37). In addition to astrocyte proliferation, these growth factors are also mitogenic to cerebellar neuronal cells (11), suggesting autocrine and paracrine mechanisms underlying the proliferative T3 mode of action.

The main finding of the present study is the demonstration that astrocytes secrete a cocktail of growth factors that mediate the mitogenic effect of T3 on cerebellar astrocytes and C6 cells. BrdU and Sytox green
incorporation clearly point to the proliferative activity of T₃ and astrocyte T₃CM instead of cell survival.

T₃ induces cerebellar astrocytes to secrete a combination of growth factors, among them bFGF, aFGF, TNF-β, and IL-3, that are partially responsible for the astrocyte and C6 cell proliferation. These growth factors may act coordinately. Therefore, bFGF seems to be the principal mitogenic growth factor secreted by cerebellar astrocytes after T₃ stimulation. However, some antibodies (anti-bFGF, anti-aFGF, and anti-TGF-β) inhibited the astrocyte or C6 cell proliferation in a mechanism independent of the T₃ action, suggesting that the basal secretion of these growth factors by astrocytes may occur.

It was reported that TNF induces astrocyte proliferation in a time- and dose-dependent manner, accompanied by downregulation of GFAP mRNA (24). TNF is also mitogenic to C6 glioma cells, increasing the expression of TNF type II receptor (16). Recently, TNF-β (also known as lymphotoxin-α) was shown to be involved in the T₃ effects on neuronal proliferation (11). In our experiments, anti-TNF-β markedly reduced astrocyte, but not C6 cell, proliferation. On the other hand, Frei et al. (8) reported an IL-3-like factor that was produced by astrocyte exposure to lipopolysaccharide. However, there is a divergence concerning IL-3 production in the central nervous system (17, 25). Our results could suggest an involvement of IL-3 on the T₃ mode of action, because the anti-IL-3 antibody significantly inhibited C6 cell proliferation without effects on astrocytes. However, this finding must be further explored. The differential effects of the neutralizing growth factor antibodies on C6 cells and cerebellar astrocyte proliferation may be explained by differences in the metabolism of these two cell types.

The members of the FGF family are found in developing and adult rat brain and are heparin-binding growth factors (27). bFGF affects the proliferation and differentiation of glial precursor cells (38) and, in vitro, stimulates the proliferation and migration of neonatal rat astrocytes (6, 15). The expression of bFGF has been shown to be elevated in tissue specimens from gliomas, meningiomas, and metastatic brain tumors (23), and antisense oligonucleotides to bFGF can inhibit the growth of transformed human astrocytes (10). C6 cells (30) and cerebellar astrocytes (12) have been demonstrated to synthesize bFGF protein. Furthermore, aFGF and bFGF are suggested to regulate both the activation and inactivation of T₃ metabolism in brain (28).

It has been reported that the activity of FGF is modulated by extracellular matrix molecules (9, 31) and that the high amounts of negatively charged polysaccharides impair interaction of growth factors with their receptors on the cell surface (1, 2). It is well known that hyaluronic acid production is high during cell proliferation and migration (33) and in the developing brain (26). In our experiments, the T₃CM from C6 cells was ineffective in promoting cell proliferation. The digestion by hyaluronidase recovered its mitogenic activity, which was impaired by anti-bFGF or heparitinase III digestion. These data suggest that T₃ stimulates bFGF secretion in C6 cells and large amounts of hyaluronic acid in C6 cultures, interfering with the growth factor mitogenic activity. The digestion of heparan sulfate chains by hyaluronidase activity also affected C6 proliferation, possibly because heparan sulfate chains are crucial to bFGF activity (40) and bind to hyaluronic acid (9). Our results may suggest the occurrence of a bFGF reservoir in the extracellular matrix through proteoglycans and hyaluronic acid in C6 cell cultures after T₃ treatment.

Previous studies have shown that heparin increases the affinity of bFGF for its receptor (31) and, in excess, competes for the binding sites within the signaling complex, and receptor transphosphorylation is reduced (18). In our experiments, we could demonstrate the stimulatory and inhibitory effects of heparin on T₃-mediated C6 proliferation. Endogenous heparan sulfate may modulate the action of the FGF secreted by T₃ stimulation in C6 cultures.

The presence of bFGF in both astrocyte and C6 T₃CM was confirmed by ELISA. T₃ stimulation increased eight times the bFGF secretion by astrocytes and two times by C6 cells. However, C6 CCM presented a basal concentration of bFGF that may be responsible for the C6 basal proliferation. The lower amount of bFGF in C6 T₃CM may explain its lower proliferative effect. Our data strongly suggest that bFGF is the main growth factor that mediates the T₃ mitogenic effect in cerebellar astrocytes and C6 cells. At least in C6 cells, its mitogenic activity may be directed by extracellular molecules such as heparan sulfate and/or hyaluronic acid.

We thank the late Prof. Carlos Chagas for constant encouragement while this work was being done. Special thanks to M. C. Fialho de Mello for helpful comments on the manuscript and to Rosenilde C. H. Afonso for technical assistance.

This work was supported by the Third World Academy of Sciences, Conselho Nacional de Desenvolvimento Científico e Tecnológico, Financiadora de Estudos e Projetos, Fundo para Pesquisa/Fundoação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro, and Prógrama de Núcleos de Excelência-Ministerio de Ciencia e Tecnologia.

REFERENCES


