Tumor Necrosis Factor as an Autocrine Growth Factor for Neuroblastoma

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ABSTRACT

Recombinant tumor necrosis factor (TNF) stimulates the proliferation of two neuroblastoma cell lines, SKNFI and SKNBE, in both serum-free medium and fetal calf serum-supplemented medium but has no effect in medium without insulin. This effect is very similar with TNF doses ranging from 5 to 500 ng/ml but depends on the duration of treatment; when cells are treated for 168 h with TNF, the maximal index of proliferation is observed between 120 and 144 h of treatment. The two neuroblastoma cell lines express type A and type B TNF receptors and contain TNF protein; however, TNF is undetectable in culture supernatants. Treatment of the two neuroblastoma cell lines with a rabbit polyclonal antibody to TNF for 96 h fully inhibits DNA synthesis. These results have been confirmed on two other neuroblastoma cell lines, IRM32 and CLB-PE.

INTRODUCTION

NB, a tumor of neuroectodermal origin which generally arises in the adrenal medulla or sympathetic chain, is the most frequent tumor in children below 5 years of age. The prognosis of this disease depends on its clinical presentation (local or metastatic spread and age of the children at diagnosis) and the cellular and molecular characteristics of the tumor (1–5). In typical stage 4 metastatic NB, the overall survival rate does not exceed 20% at 5 years although major progress has recently been achieved, both in the induction of antitumoral response to first line chemotherapy and in the consolidation with megatherapy (6). Both the spontaneous regression of the tumor, observed in infants, and the ability of NB to differentiate in vitro and in vivo favor the hypothesis that new therapeutic approaches based on the use of biological response modifiers may improve the prognosis of metastatic NB.

Most current clinical protocols using biological response modifiers are based on the stimulation of the immune system with various cytokines used alone or in combination. None of these cytokines, however, has a univoque (single-targeted) effect and several of them have been shown to modulate malignant cell proliferation in vitro. TNF, a monokine produced by activated macrophages, mediates antitumor effects in vitro through a broad spectrum of activities. Its cytotoxic or cytostatic properties in vitro on malignant cells and cell lines and the regression of some animal tumors, as reviewed by Semenzaio (7), have led to investigations of its therapeutic value in clinical oncology. However, recent studies have clearly shown that TNF stimulates the growth of normal nonhematopoietic tissues as well as hematopoietic ones (8, 9). Furthermore, TNF is an autocrine growth factor for B-cell chronic malignancies (10), and it has also been shown to enhance the proliferation of an astrocytoma cell line (11).

In this study we demonstrated that TNF enhanced the proliferation of two NB cell lines, SKNFI and SKNBE; TNF protein is detected in the two cell lines and a rabbit polyclonal antibody to TNF or two monoclonal antibodies against type A and type B TNF receptors fully inhibit DNA synthesis. These results have been confirmed on two other NB cell lines.

MATERIALS AND METHODS

Human Cell Lines and Stock Cultures, Normal Tissues

All cell lines were checked for the absence of Mycoplasma.

NB Cell Lines. SKNFI and SKNBE neuroblastoma cell lines used in this study were kindly provided by Dr. L. Helson (Sloan Kettering Memorial Hospital, New York, NY). IRM32 was obtained from the American Type Culture Collection; CLB-PE was recently established from a bone marrow metastasis harvested at relapse in one of our patients with stage 4 NB and has been successfully passaged in nude mice. The characteristics of SKNFI, SKNBE, and IRM32 have been described previously (12–14). The four cell lines displayed N-myc amplification. NB cell line stock cultures were grown in monolayer culture, either in serum-containing medium (RPMI 1640; Gibco, Cergy-Pontoise, France) containing 10% heat-inactivated FCS (Fllobio, Courbevoie, France) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mm glutamine or in a serum-free medium (Ham’s F-12 medium; Dulbecco’s modified Eagle’s medium, 1:1; Gibco) supplemented with 100 μg/ml transferrin, 5 μg/ml insulin, 20 nM progesterone, 100 μM putrescine, 30 μM selenium, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mm glutamine, as described previously for NB cell culture (15).

In cultures with serum-containing medium, SKNFI cells are highly substrate adherent, with a fibroblast-like appearance, growing in swarming arrays without apparent directional orientation. SKNFI, CLB-PE, and IRM32 cultured in serum-containing medium grew as undifferentiated small, round, and blue cells with scanty cytoplasm, occasional long cellular processes, and weak adhesiveness to the surface. Culture of NB cell lines in serum-free medium induced, as described previously, the neuritic differentiation of neuroblasts which acquired long cellular processes (15).

Other Cell Lines. HL60, a promyelocytic cell line, LAZ 509, a lymphoblastoid B-cell line, and CAKI, a renal carcinoma cell line, were obtained from the American Type Culture Collection. Daudi, a melanoma cell line, was kindly provided by Dr. J. F. Doré (Inserm U.212, Lyon, France). These lines were maintained in RPMI 1640 supplemented with 10% FCS and used as positive or negative controls during experiments.

Normal Tissues. Peripheral blood and bone marrow mononuclear cells (Ficoll separation after aspiration on heparin) and platelets (separation by low centrifugation, 15 min at 200 × g) were obtained from normal volunteers and used as negative controls.
Recombinant Cytokines and Antibodies

rTNF with over $5 \times 10^5$ units/mg specific activity (as measured with a bioassay) was generously provided by Eurocetus (Amsterdam, the Netherlands). Mouse monoclonal antibodies (IgG1 isotype) against type A (M, 75,000) TNF receptor (UTR1) and type B (M, 55,000) TNF receptor (HTR5 and HTR9) were kindly provided by Dr. Manfred Brockhaus (Hoffman-LaRoche, Basel, Switzerland) (16). HTR9 has TNF-like activity, whereas UTR1 and HTR5 are TNF antagonists for type A and type B TNF receptors, respectively (17-19). These antibodies were used at the concentration of 10 µg/ml for immunocytological detection and at 50 µg/ml in inhibition experiments (16-19).

A mouse monoclonal antibody against TNF (clone TNF3; Tebu, Le Perray en Yvelines, France) was used at 1:20 dilution for immunological detection of TNF in NB. A rabbit polyclonal antibody to TNF (clone 8926; M. Brockhaus, Hoffman-LaRoche) which has been shown to block the binding of TNF to its receptors and its biological activities, was used in inhibition experiments (20).

Proliferation Assays and [3H]Thymidine Incorporation

All experiments were performed with NB cells grown as stock cultures in appropriate medium.

After division and medium change, cells from the stock culture were seeded on cell plates and cultured for 18 h (5% CO₂ and 37°C) to allow cell growth and attachment before starting the assay. Then, on the first day of the assay, the medium was removed by aspiration and replaced by fresh medium supplemented with the factor to be tested at appropriate dilution (rTNF, polyclonal antibody to TNF, or monoclonal antibodies against TNF receptors). Cells were cultured (5% CO₂ and 37°C) up to 96 or 168 h. In prolonged cultures, the medium was removed by aspiration at 96 h and replaced by fresh medium with reagents used at the same dilution.

[3H]Thymidine Incorporation. DNA synthesis was evaluated by measuring [3H]thymidine incorporation. Cells were seeded in 96-well plates (Falcon) (10⁶ cells in 200 µl/well); 3 to 5 replicate wells were used for each experimental condition. [3H]Thymidine (CEA, Gif sur Yvette, France; specific activity, 1 Ci/mmol) was added to the wells (0.5 µCi/well) and incubated with the cells for 18 h before measurement. Cells were then lysed by two freezing and thawing cycles and harvested on glass fiber filters (Skatron-USA, Os, France) with a semi-automatic cell harvester (Skatron-USA); the cellular [3H]thymidine uptake was determined in liquid scintillation fluid (Pico-fluor TM30; Packard Instruments SA, Rungis, France) with a β-scintillation counter (Tri-Carb; Packard).

Determination of Cell Proliferation. Cell number and viability after exposure to TNF were determined by cell counting and trypan blue exclusion (Gibco) of dead cells. Cells were seeded in 24-well tissue culture plates (Falcon; Becton-Dickinson, Grenoble, France) (5 x 10⁴ cells in 200 µl/well); 3 to 5 replicate wells were used for each experimental condition. [3H]Thymidine (CEA, Gif sur Yvette, France; specific activity, 1 Ci/mmol) was added to the wells (0.5 µCi/well) and incubated with the cells for 18 h before measurement. Cells were then lysed by two freezing and thawing cycles and harvested on glass fiber filters (Skatron-USA, Os, France) with a semi-automatic cell harvester (Skatron-USA); the cellular [3H]thymidine uptake was determined in liquid scintillation fluid (Pico-fluor TM30; Packard Instruments SA, Rungis, France) with a β-scintillation counter (Tri-Carb; Packard).

Analysis of Binding Sites for TNF

Radioligands. [125]Iodotyrosyl-TNF, purchased from Amersham France SA, had a specific radioactivity of about 800 Ci/mmol.

Binding assay for TNF. A competition displacement assay was performed. Cells were harvested with PBS-EDTA, washed once, and resuspended at 1x10⁶ cells in 100 µl of PBS supplemented with 0.1% bovine serum albumin and 0.02% sodium azide and were incubated for 4 h at 4°C with 0.07 nM [125]I-rTNF alone or in the presence of increasing concentrations of unlabeled recombinant human TNF (0.03 nM to 1.5 nM). Nonspecific binding was determined in parallel incubations with a 200-fold excess of unlabeled rTNF.

After incubation cells were centrifuged for 5 min and unbound [125]I was aspirated. The cell pellets were washed twice with cold PBS supplemented with 0.1% bovine serum albumin buffer. The amount of bound and free [125]I was determined by counting the cell pellets and supernatants in a gamma counter (Packard Instruments SA).

The affinity constants and the number of receptors were calculated from the binding curves by a Scatchard analysis.

Immunocytological Detection of Type A and Type B TNF Receptors and TNF Protein on NB Cells

Alkaline Phosphatase Immunostaining. NB cells were detached with PBS-EDTA from stock culture in phase of exponential growth (72 h after division and medium change); they were resuspended in phosphate-buffered saline (Gibco) at 10⁶ cells/ml and spin down on centrifuged smears (10⁵ cells/smear). Immunocytochemical staining was performed using an indirect 3-stage immunoenzymatic procedure with alkaline phosphatase (Dakopatts, Copenhagen, Denmark), as already described (21). Briefly, air-dried slides (cytocentrifuged smears) were fixed for 5 min with acetone at 4°C and incubated for 60 min with monoclonal antibodies, then for 30 min with enzyme-conjugated rabbit anti-mouse immunoglobulins (Dakopatts), and for 30 min with enzyme-conjugated swine anti-rabbit immunoglobulins (Dakopatts). Washes were done with Tris buffer. The final step consisted of a 15-min incubation with Naphthol-As-Mx phosphate, dimethylformamide, levamisole, and fast red (Sigma-USA, Grenoble, France). Slides were counterstained with hematoxylin, mounted permanently with glycerin, and evaluated under an optical microscope.

Quantification of TNF in Cell Pellet Lysates and Culture Supernatant

The quantification of TNF and lymphostatin present in culture supernatants and cell pellets was assayed by enzyme-linked immunosorbent assay (Genzyme-USA; Tebu, for TNF; Immunotech, Marseille, France, for lymphostatin). Culture supernatant was removed at different times of the culture and stored at —70°C.

For preparation of cultured cell lysates, cells were detached by PBS-EDTA (0.5 mm) from stock cultures in exponential phase of growth (72 h after division and medium change); they were counted and washed twice with sterile phosphate-buffered saline and centrifuged. Pellets of 6 x 10⁶ cells/ml were then sonicated on ice during 1 min with a 2000S sonicator (Bioblock Scientific, Strasbourg, France) and stored at —70°C until TNF assay. The concentration of TNF was thus expressed in pg/ml or in pg/µg of proteins in the cell pellets. Total proteins were quantified by the Bio-Rad protein colorimetric assay (Bio-Rad, Paris, France).

RESULTS

Growth-promoting Effect of TNF on Two NB Cell Lines, SKNFI and SKNBE. TNF stimulated DNA synthesis in NB cells with a lag period of 96 h and a maximal effect observed between 120 and 144 h. One typical experiment for each cell line is shown in Fig. 1. TNF stimulation was observed at 5 ng/ml (or 25 IU/ml) and its enhancing effect was very similar at 50 and 500 ng/ml. The maximal index of stimulation (number of cpm in treated samples on number of cpm in control cultures) obtained in three different experiments reached mean values of 1.30, 1.41, and 1.45 for SKNFI cultured with 5, 50, and 500 ng/ml TNF, respectively (range, 1.04—1.95); it reached mean values of 1.25, 1.36, and 1.32 for SKNBE cultured with 5, 50, and 500 ng/ml TNF, respectively (range, 1.02—2.02).

The stimulation of [3H]thymidine incorporation by TNF was very similar on the two NB cell lines cultured in serum-free medium supplemented with insulin, but TNF had no effect in serum-free medium without insulin (data not shown).

The effect of TNF on NB cell proliferation and viability was measured by counting cells with trypan blue exclusion in cultures from 96 to 144 h (Table 1). An 80% or more viability was observed in all tested conditions (time and TNF concentration); it was equal to that observed in untreated samples. Yet, TNF at concentrations ranging from 5 to 500 ng/ml stimulated...
SKNFI and SKNBE NB cell proliferation in serum-free and FCS-supplemented medium, with a maximal effect usually observed at 144 h.

Examination of cell cultures showed that TNF maintained cell integrity. Under treatment with TNF, malignant cells recovered morphological characteristics of undifferentiated small round and blue cells with scant cytoplasm; they lost long cellular processes which are typically observed on untreated SKNBE cell line, whether it is cultured in serum-free medium or in FCS-supplemented serum, and on untreated SKNFI cell line when it is cultured in serum-free medium.

Expression of TNF Receptors on SKNBE and SKNFI NB Cell Lines. Upon Scatchard analysis, SKNFI had 250 binding sites/cell (K_d 1.5 x 10^{-10} M) in serum-containing medium and 550 in serum-free medium supplemented with insulin, without variation of affinity.

SKNBE had 308 binding sites per cell with an apparent K_d of 1.5 to 1.6 x 10^{-10} M, without variation with the composition of the medium.

The immunocytochemical analysis of the two M, 55,000 (type B) and M, 75,000 (type A) receptors with monoclonal antibodies showed that SKNFI and SKNBE expressed both receptors. A stronger positivity was observed with UTR1 (type A receptors) than with HTR9 (type B receptors) (see Fig. 2A).

Inhibition of NB Cell Proliferation by a Polyclonal Antibody to TNF. As shown in Fig. 3, treatment of NB cells with a rabbit polyclonal antibody to TNF during 72 h blocked [3H]thymidine incorporation in both cell lines. The effect was maximal at the lowest antiserum dilution tested (1:5) on SKNFI, whereas over 95% inhibition was observed with all antiserum dilutions (1:5-1:50) on SKNBE.

We then measured, from 48 to 96 h, the effect of the antiserum to TNF used at the lowest dilution (1:5) on both cell lines (Table 2). At 96 h, over 95% inhibition of [3H]thymidine incorporation was observed for both cell lines in two different experiments; the cell viability was inferior to 5%.

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**Table 1 Stimulation of neuroblastoma cell proliferation by TNF**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>5 ng/ml</th>
<th>50 ng/ml</th>
<th>500 ng/ml</th>
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<td>Serum-free medium</td>
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<td></td>
</tr>
<tr>
<td>SKNFI</td>
<td>Experiment 1</td>
<td>1.51</td>
<td>3.30</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>1.22</td>
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<td>1.67</td>
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<tr>
<td>Experiment 3</td>
<td>2.10</td>
<td>2.57</td>
<td>1.71</td>
</tr>
<tr>
<td>SKNBE</td>
<td>Experiment 1</td>
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<td>1.10</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>1.85</td>
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<td>NT</td>
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<td>Experiment 3</td>
<td>1.65</td>
<td>1.49</td>
<td>2.76</td>
</tr>
<tr>
<td>FCS-supplemented medium</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SKNFI</td>
<td>Experiment 1</td>
<td>1.83</td>
<td>1.97</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>1.55</td>
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<td>Experiment 3</td>
<td>1.31</td>
<td>1.82</td>
<td>1.51</td>
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<tr>
<td>SKNBE</td>
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<td>1.22</td>
<td>1.64</td>
</tr>
<tr>
<td>Experiment 2</td>
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<td>NT</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>1.80</td>
<td>2.10</td>
<td>1.40</td>
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</table>

* Number of viable cells in treated samples on number of viable cells in control culture.

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Inhibition of SKNFI and SKNBE NB Cell Proliferation by Monoclonal Antibodies against Type A and Type B TNF Receptors. As shown on Fig. 4, [3H]thymidine incorporation by SKNFI and SKNBE cell lines is inhibited by monoclonal antibodies directed against type A and type B TNF receptors; maximal effect is observed at 96 h when the two monoclonal antibodies are used in combination. UTR1 (against type A receptor) used as single agent has no effect on SKNFI and induces only 50% inhibition of [3H]thymidine incorporation on SKNBE; however, it synergizes with HTR5 to induce maximal

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**Fig. 1.** TNF stimulation of [3H]thymidine incorporation by SKNFI and SKNBE neuroblastoma cell lines. (O) no TNF; (O) 5 ng/ml TNF; (O) 50 ng/ml TNF; (*) 500 ng/ml TNF.

Cells were seeded in 96-well culture dishes (1 x 10^5 cells in 200 μl) after division and medium change. They were cultured for 18 h to allow for growth and attachment. Then the medium was removed by aspiration, and fresh medium containing TNF at various concentrations was added to the wells. Cells were cultured for up to 168 h; the medium supplemented with TNF was supplemented at 168 h; the medium supplemented with TNF at the same dilution was changed at 96 h. On both cell lines thymidine incorporation dramatically decreased in controls and in treated samples at 168 h, unless the medium was changed and cells were divided. Results are thus validly interpretable up to 144 h in conditions described here. Peak proliferation was usually observed at 144 h; it also occasionally occurred at 120 or 168 h. Results represent the index of proliferation obtained on the day of peak proliferation.
Fig. 2. Immunocytological detection of TNF receptors and TNF protein in SKNFI and SKNBE neuroblastoma cell lines. A1, A2, and A3, type A TNF receptor on SKNBE, SKNFI, and HL60, respectively; A4, A5, and A6, type B TNF receptor on SKNBE, SKNFI, and HL60, respectively. Type A receptor is recognized by UTR1 and type B receptor is recognized by HTR9. HL60, a promyelocytic cell line, was used as positive control. The pattern of reactivity on the two neuroblastoma cell lines was similar to that observed on HL60, with a stronger reactivity with UTR1 than with HTR9, as reported previously (16). Normal human bone marrow mononuclear cells obtained after Ficoll separation of the whole aspirate were used as negative control; no staining was observed. B1, B2, and B3, TNF expression in SKNBE, SKNFI, and LAZ 509, respectively. TNF protein was detected with an anti-TNF monoclonal antibody (clone TNF3). The human lymphoblastoid B-cell line LAZ 509 was used as positive control. One renal cell carcinoma cell line was used as negative control; no staining was observed. Original magnification, × 40.
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Fig. 3. Inhibition of [3H]thymidine incorporation in SKNFI and SKNBE neuroblastoma cell lines by a rabbit antiserum at dilutions of 1:5, 1:10, 1:20, and 1:50 directed against TNF. O, control serum (rabbit serum at the highest concentration) (dilution 1:5); Control, untreated control. Cells were cultured in serum-containing medium. Eighteen h after seeding, the culture medium was replaced with fresh medium containing a polyclonal antibody to TNF, or normal rabbit serum at the same dilution. Cells were then cultured for 72 h. Results are expressed as a percentage of the [3H]thymidine incorporation in experimental groups compared to untreated control samples. Each result represents mean ± SEM (bars) of triplicate determinations. The anti-TNF used at the lowest dilution (1:5) had no effect on the cell growth of a renal carcinoma cell line and a melanoma cell line used as negative controls. These results rule out the possibility of a nonspecific toxicity of this reagent on cells.

Table 2 Kinetics of neuroblastoma cell growth inhibition by a rabbit antiserum against TNF

See legend to Fig. 3 for experimental conditions and negative controls. Polyclonal antibody against TNF was used at 1:5 final dilution. Results are mean ± SEM obtained in two distinct experiments with triplicate wells in each experiment.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>[3H]Thymidine uptake (cpm x 10^-3)</th>
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<td></td>
<td>48 h culture</td>
</tr>
<tr>
<td>SKNBE</td>
<td></td>
</tr>
<tr>
<td>Untrained control</td>
<td>18.6 ± 1.6</td>
</tr>
<tr>
<td>Antibody against TNF</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>Rabbit serum</td>
<td>19.3 ± 1.1</td>
</tr>
<tr>
<td>SKNFI</td>
<td></td>
</tr>
<tr>
<td>Untrained control</td>
<td>74.3 ± 10.6</td>
</tr>
<tr>
<td>Antibody against TNF</td>
<td>8.9 ± 1.3</td>
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<tr>
<td>Rabbit serum</td>
<td>75.6 ± 8.4</td>
</tr>
</tbody>
</table>

inhibition of [3H]thymidine incorporation, suggesting that the two receptors are functional.

Detection of TNF Protein in the Two NB Cell Lines. As shown on Fig. 2, B1 and B2, immunocytochemical staining with a monoclonal antibody against TNF enabled detection of the presence of cytoplasmic and perimembranar TNF in over 90% of the cells in both lines.

We thus measured TNF protein, using a solid phase enzyme-linked immunosorbent assay, in culture supernatants and cell lysates of SKNFI and SKNBE. TNF was never detected in the supernatant of NB cell lines but was present in cell pellets. The mean quantity of TNF protein was 48 ± 6.1 pg for 6 x 10^6 SKNBE cells, and 48.1 ± 4.1 pg for 6 x 10^6 SKNFI cells (mean, 4 experiments). LAZ 509 used as positive control in the same conditions contained 38.7 ± 13.3 pg of TNF for 6 x 10^6 cells, although it secreted 97.8 ± 8.3 pg/ml in culture supernatant.

TNF was also quantified in pg/μg of total proteins in the cell pellets. SKNBE and SKNFI contained 11.8 and 5.44 pg of TNF/μg of protein, respectively, and LAZ 509 contained 18.22 pg/μg. Peripheral blood lymphocytes and platelets, used as negative controls, contained less than 1 pg/μg; one renal carcinoma cell line and one melanoma cell line were tested in the same conditions and contained 2 pg TNF/μg of protein or less. Lymphotoxin was undetected in the two NB cell lines.

TNF as an Autocrine Growth Factor on Two Other Cell Lines, IRM32 and CLB-PE. Results obtained on SKNFI and SKNBE were confirmed on two other NB cell lines, IRM32 and CLB-PE; the latter was recently established from the bone marrow metastasis harvested at relapse in one of our patients with stage 4 NB.

TNF used at the dose of 5 ng/ml stimulated the proliferation of both cell lines; the maximal index of proliferation observed at 144 h was 1.7 for IRM32 and 1.65 for CLB-PE. TNF was undetectable in the supernatant of the two cell lines but the cell...
pellet from IRM32 contained 77.8 pg TNF/μg of protein, and CLB-PE contained 32.4 pg/μg of protein.

The treatment of both cell lines with a combination of the two monoclonal antibodies against type A and type B receptors during 96 h induced over 90% inhibition of [3H]thymidine incorporation; the cell viability in the cultures was inferior to 5%. UTR1 (anti-type A receptor) used alone induced only 23% inhibition of [3H]thymidine incorporation on IRM32 and 60% on CLB-PE at 96 h. HTR5 (anti-type B receptor) induced 58% inhibition of [3H]thymidine incorporation on IRM32 and 74% on CLB-PE.

**DISCUSSION**

TNF can be produced by normal hematopoietic tissues and their malignant counterpart as well as some nonhematopoietic cells (22). In particular, TNF has been detected in low grade B-cell non-Hodgkin’s lymphoma, B-CLL, hairy cell leukemia, and acute myeloid leukemia, as well as in colon carcinoma cell lines (10, 23–26). TNF clearly acts as an autocrine growth factor for chronic B-cell malignancies (10), whereas its effect is more complex in acute myeloid leukemia (10, 27, 28). TNF also promotes the growth one human astrocytoma cell line (11).

In this study we demonstrated that TNF stimulates the in vitro proliferation of four well-characterized NB cell lines. NB cells contain TNF protein, and a rabbit polyclonal antibody to TNF fully blocks DNA synthesis and survival of the cells. Inhibition experiments with type A and type B receptor-specific antibodies showed that both receptors are functional. A single monoclonal antibody against type A or type B receptors does not, or only partially, block NB growth, whereas the combination of both induced over 85% growth inhibition.

Thus, TNF is a new example of well-defined factor that can act in both the immune and the neural system. This observation raises important questions of biological and clinical significance.

For the biologists, a first question is to know whether or not TNF may interact with other growth factors on NB cells. As described previously for normal fibroblasts, the growth-promoting activity of TNF is observed in serum-free medium, but it is dependent on the presence of insulin in the medium (8). These results, as well as previous reports on the autocrine growth activity of IGF-II on a NB cell line, suggest that insulin or related factors may act as cofactors for TNF (29).

The molecular cloning of two TNF receptors and the analysis of amino acid sequences has revealed a large homology with the cystine-rich extracellular domains of the nerve growth factor receptor (30). Nerve growth factor receptor is expressed by the four NB cell lines (data not shown). Although the coexpression of these molecules does not imply their interaction, one will favor the idea that their association on neuroblastoma is not fortuitous. NB cell lines may be used to analyze the relationship between the two receptors.

Several other cytokines may regulate the expression and/or the activity of TNF on NB, including γ-interferon, interleukin 1α and interleukin 6. We are currently investigating in the NB model the putative interaction of TNF with these different growth factors.

A second question arises from the observation that TNF protein is present in cell lysates but is undetectable in culture supernatants. Similarly, Cordingley et al. (10) did not detect TNF in the supernatants of cultured HCL/B-CLL; however, while fresh cells contain barely undetectable TNF mRNA, they showed that TNF protein greatly enhances TNF message levels. We currently analyzed this phenomenon in NB cells. Although secretory TNF may be inhibited by soluble TNF receptors in culture supernatants, this hypothesis is very unlikely, since soluble TNF receptors are undetectable in culture supernatants of the NB cell lines (data not shown). In the neuroblastoma model as in B-CLL, transmembranar TNF may stimulate the growth of malignant cells. If so, the growth-promoting activity of transmembranar TNF on NB cells will then require cell-cell contact and may thus be considered as a paracrine stimulation on neighboring cells.

A third issue would be to know whether our observations can be extended to the rare cell lines which do not present N-myc amplification. Indeed, we demonstrated that TNF is an autocrine growth factor for 4 NB cell lines with N-myc amplification. It has recently been shown that enhanced N-myc expression contributes to malignant progression of human NB cells, conceivably by stimulating the activity of an unidentified autocrine growth factor (31).

For the clinicians, one major issue is to know whether or not our observation may be extended to in vivo situations, in particular if one considers using biological response modifiers in the treatment of neuroblastoma.

Results presented here should imply that the administration of TNF to patients with NB would have adverse effects and that the alternative therapy is the injection of TNF antagonists. Furthermore, we previously showed that a systemic injection of interleukin 2 induced TNF secretion in vivo (32); whether this observation can explain the lack of clinical response to interleukin 2 therapy in patients with end stage NB must be investigated (33). We currently analyze the expression of TNF protein and TNF receptors on fresh tumoral specimens obtained from our patients, as well as the effect of TNF or its antagonists on primary NB cultures.

**ACKNOWLEDGMENTS**

Polyclonal antibody against TNF and monoclonal antibodies against TNF receptor were kindly provided by Dr. Manfred Brockhaus (Hoffmann-LaRoche, Basel, Switzerland).

**REFERENCES**


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