FACTORS RELEASED BY RAT TYPE 1 ASTROCYTES EXERT DIFFERENT EFFECTS ON THE PROLIFERATION OF HUMAN NEUROBLASTOMA CELLS (SH-SY5Y) "IN VITRO".

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ABSTRACT

Brain metastasis derived from abdominal neuroblastoma are an uncommon complication of this tumour; however, an increase of their incidence has been recently reported. In this study, we have investigated the influence of factors derived from CNS glial cells on human neuroblastoma cells (SH-SY5Y) proliferation "in vitro". Co-culture experiments show that a 24 h exposure to factors released by type 1 astrocytes (A1) may induce a significant decrease of [^{3}H]thymidine ([^{3}H]TdR) incorporation in SH-SY5Y cells. This effect was not duplicated by fresh A1-conditioned medium (A1-CM); A1-CM become active only when it was heated or frozen. In contrast with this short-lived inhibitory effect, a long-term treatment (3, 6, and 9 days) with A1-CM produces a significant and dose-dependent increase of SH-SY5Y cell number. Immunoneutralisation of A1-CM with an anti-TGFß antibody eliminates the inhibitory effect on [^{3}H]TdR uptake in SH-SY5Y cells, but it does not affect the increased number of viable cells observed after long term treatments.

In conclusion, these results show that factor(s) released by A1 may affect the proliferation/survival of a human neuroblastoma cell line "in vitro" inducing: a) a short transient negative effect on DNA synthesis; and b) an overall sustained trophic action. These results are suggestive of a possible role of glial cells in the establishment of brain metastases of neuroblastomas.

INTRODUCTION

Neuroblastomas are solid tumours of childhood characterised by an undifferentiated cell phenotypes and a poor prognosis. Occasionally, these tumours show spontaneous regressions due to their differentiation into benign ganglioneuromas. Unfortunately, the mechanisms which drive this differentiation are still poorly understood. The neuroblastoma is usually detected as a solid abdominal mass with diffuse metastasis, although invasion of brain tissue was believed to be a rare manifestation of extra- or intra-cranial neuroblastoma. However, it has recently reported that 68% of patients affected by neuroblastoma or ganglioneuroblastoma suffer of neurological complications due to undiagnosed or hidden brain metastases (Weyl-Ben Arush et al., 1995; Tasdemiroglu and Patchell, 1997). These lesions may appear as a relapse after chemotherapy, and they may become evident after the disappearance of tumoral tissue from the usual localisation(Shaw and Eden, 1992; Astigarra et al., 1996; Bouffet et al., 1997; Lydaki et al., 1997).

The nervous system is composed of different types of cells which include, in addition to neurons, also glial elements (e.g., astrocytes, oligodendrocytes, Schwann cells, etc.). In the last few years, it became evident that glial cells may play a relevant role in modulating several neuronal functions. Glia-neuron interactions were shown to control neuronal migration and differentiation, to provide spatial and metabolic support to neurons, and to modulate their synaptic activity (Banker, 1980; Lindsay, 1987; Hatten and Mason, 1990; LoPachin and Aschner, 1993). Glial cells, and astrocytes in particular, were found to release many factors (e.g., neurotransmitters, neuropeptides, cytokines, growth factors, etc.) known to possess trophic or differentiating activities on neuronal and non-neuronal cells (Giulian et al., 1988; Muller et al., 1995). Consequently, the establishment of brain metastases of tumoral cells might also depend, at least in part, on the trophic support provided by glial elements.

Many studies have shown that the conditioned media (CM) in which glial cell were growth may generally induce neuritogenesis and a neuron-like morphological differentiation of neuroblastoma cells. Even though these results appear in apparent contrast with the possibility of an establishment of brain metastases of these tumours, the data concerning the effects of secretory products of the glial cells on the proliferation of neuroblastoma cells (Monard et al., 1973; Kato et al., 1983; Sakazaki et al., 1983; Lim et al., 1990; Amano et al., 1994) appear sometime incomplete. The reasons of such discrepancies may reside in the different experimental conditions adopted. For instance, a number of these studies have been performed using the CM obtained from tumoral glial cells (e.g., glioma cells) rather than from untransformed astrocytes; moreover, it has recently reported that the glial factors involved in the differentiation and neuritogenesis of neuroblastoma cells are apparently released only from young and sub-confluent astrocytes (Shea et al., 1994). On the other

, the presence of brain metastases may be suggestive of a possible role of the glial elements on the survival and/or proliferation of neuroblastoma cells. Also considering that astrocytes "in vitro" may behave differently from those "in vivo", the utilisation of cells obtained from primary cultures may give useful information on the trophic factors physiologically produced and released by these glial cells. Therefore, the aim of the present study was to investigate whether cultures of neonatal type 1 astrocytes, might produce and release "in vitro" some factor(s) which might exert trophic activity on neuroblastoma cells.

The experiments have been conducted using the human neuroblastoma cell line SH-SY5Y (Biedler et al., 1973); these cells have been subcloned from the parental SK-N-SH cell line, obtained from a bone marrow metastasis of a neuroblastoma, and have been previously extensively characterised (Pahlman et al., 1990).

MATERIALS AND METHODS

Cell cultures

Primary cultures of type 1 and type 2 astrocytes were obtained from the cerebral cortex of 1- to 2-day old rats as previously described (Melcangi et al., 1993). Human SH-SY5Y neuroblastoma cells (kindly provided by Dr. June Biedler, Memorial Sloan-Kettering Cancer Center, New York) were derived from the parental SK-N-SH cell line which was established from a bone marrow metastasis of a 4-year old girl (Biedler et al., 1973). The cells were grown at 37°C in a humidified CO_2 incubator in Minimum Essential Medium supplemented with non-essential aminoacids, 1 mM sodium pyruvate, 100 μg streptomycin/ml, 100 UI penicillin/ml, 10 mg/l of phenol red (Biochrom KG, Berlin, Germany) and 10% foetal bovine serum (FBS, Gibco, Grand Island, NY). Confluent cells were harvested with 0.05/0.02% trypsin/EDTA and 1.10^6 cells were seeded in 57 cm^2 dishes. The medium was replaced at 2-day intervals.

The neutralising pan-specific anti-TGFß antibody (directed to r(h)TGFß1, 1.2, 2, 3 and 5; R&D System, Minneapolis; cat.# AB-100-NA) was diluted according to the manufacturer's instructions.

Collection of conditioned media Glial cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Biochrom, KG, Berlin, Germany), supplemented with 10% FBS, 100 μg streptomycin/ml and 100 UI penicillin/ml (Melcangi et al., 1993), until confluence was reached. To avoid interferences due to factors present in the FBS, the culture medium was replaced with Sato's chemical-defined medium (DMEM supplemented with 5 μg/ml bovine insulin, 50 μg/ml human transferrin, 20 nM progesterone, 100 μM putrescine dihydrochloride, 30 nM sodium selenite (Sigma Chemicals, St.Louis, MO) and the cell cultures were returned in the incubator; the medium (conditioned medium, CM) was collected 24 hours later and used immediately or stored at -20°C as described in the Result section. Sato's medium left in the incubator for 24 hours and stored as the CM was used as control.

Co-culture experiments Neuroblastoma cells were plated in 6-well tissue culture plates, while type 1 astrocytes were plated in 25mm cell culture insert (Falcon, Becton Dickinson Labware, England). The bottom of the insert is an opaque membrane, with a pore size of 0.45 μm which is specifically designed to provide independent access of the medium to both sides of the membrane. Cell culture inserts containing confluent purified type 1 astrocytes at 95% confluence were transferred to wells in which neuroblastoma cells were plated 24 h before. At the same time the medium was replaced with fresh Sato's defined medium .

Neuroblastoma cells cultured alone in 6-well plate and pure type 1 astrocytes cultured alone on the well insert were utilised as controls. Four hours before the end of the co-culture period, the cultures were processed for [^{3}H]thymidine incorporation and cell counting as described below.

[^{3}H]thymidine incorporation

DNA synthesis was evaluated by measuring the incorporation of radiolabelled nucleotides, in trichloroacetic acid precipitable fraction of the cells, after a short time exposure of the cells (less than the half of their doubling time) to the factors under investigation. This allows to evaluate the rate of DNA synthesis in the absence of significant modifications of cell number, which, however, is verified at the end of the incubation period. For the evaluation of the incorporation of [methyl-^{3}H]thymidine ([^{3}H]TdR) (Amersham, Milan, Italy), the cells were seeded in 24 wells/plates (50000 cells/well). Three days later the media were changed with fresh medium with or without FBS, 48 h later the medium was replaced with control medium or glial cell- conditioned media. A pulse of [^{3}H]TdR (10 μCi/ml) was added 20 hours after the beginning of the various treatments and left in the culture medium for 4 h. After then, the cells were washed with 0.5 ml of Dulbecco's PBS prewarmed to 37°C, fixed with 1 ml of ice-cold 10% trichloroacetic acid and 10 min later lysed with 0.1 ml of 6N NaOH. A parallel set of wells from the same plate were processed as for [^{3}H]TdR incorporation and the cell number evaluated at the end of the washing procedure. The cell lysate were then collected and mixed with 7 ml of scintillation cocktail (Instagel®, Packard, Milano, Italy) and counted in a scintillator for beta emitters (TriCarb 6000, Packard) having a 60% of efficiency.

Evaluation of cells viability

The number of cells, counted using an hemocytometer, and the cell viability have been taken as the measure of cell proliferation and survival in long term experiments. To this purpose, SH-SY5Y cells were seeded in 24 wells/plates (30000 cells/well) three days later the media were changed with control medium or type 1 astrocytes -conditioned medium (A1-CM). The media were changed every three days throughout the period of treatment. The amount of viable cells was measured by counting the cells with an hemocytometer after trypan blue stain or by using the diphenyl-tetrazolium salt (MTT) procedure (Manthorpe et al., 1986). Briefly, cells exposed to the various treatments were washed with warm culture medium and incubated for 1 h at 37°C with a solution of MTT (1 mg/ml)(Sigma Chemicals, St.Louis, MO) dissolved in culture medium without FBS and phenol red. The solution was then decanted and 1 ml/ well of isopropanol was added to dissolve formazan crystals; the absorbance at 560 nm, determined by a microplate spectrophotometer, and subtracted of the blank wells and of the absorbance at 630 nm was taken as an index of the number of living cells in culture. The linearity of the response ($r>0.98$) of the MTT assay ranged from 0.001 to 0.3 units of absorbance, corresponding to 5000 and 250.000 cells/well, respectively. The assay was run in triplicates (the coefficient of variation among replicates was less than 10%).

Each other result presented in this study has been obtained from 3-4 independent experiments run in 4 replicates.

Statistical analysis

The results were analysed by ANOVA and adequate post-hoc tests (Student t-test, Dunnett). The dose-response curves were analysed by means of a Macintosh version of the program ALLFIT (De Lean et al., 1978).

RESULTS

The study was initially conducted using confluent rat type 1 astrocytes (A1) and SH-SY5Y neuroblastoma cells maintained in co-culture in a two-chamber device, which permits the free exchanges of soluble factors without allowing any physical contact between the two cell populations (see Materials and Methods). To make sure that the effects observed were due directly to factor(s) released from the astrocytes, and not mediated by principles present in the foetal bovine serum (FBS), a chemical-defined medium (Sato's medium) was used throughout the experiments.

The results of the co-culture experiments are illustrated in Figure 1. In order to simplify their lecture, the data are presented as percent variations vs. the respective controls; however, all necessary statistical analyses have been performed on the absolute values. Figure 1 (panel a) shows that a 24-h co-culture with A1 significantly inhibits the rate of incorporation of [^{3}H]TdR in SH-SY5Y cells when compared to that occurring in neuroblastoma cells cultured alone in chemical-defined medium. There were no significant modifications of the number of living neuroblastoma cells, evaluated by counting the cells at the end of the 24-h of co-culture, using the trypan blue exclusion method (Fig. 1, panel b). This suggests that the observed decrease of the incorporation of [^{3}H]TdR is linked to a decrease of DNA synthesis, rather than to decrease of the cell viability due to toxic factors released by astrocytes. A more prolonged time of co-culture (48 h) with A1 allows the values of [^{3}H]TdR incorporation in SH-SY5Y cells to return to control levels (Fig. 1, panel a). After 48 h of co-culture with A1 the number of living neuroblastoma cells resulted to be significantly increased vs. controls (Fig. 1, panel b). Apparently, DNA synthesis returns to normal levels allowing cell duplication .

To reduce the complexity of the experimental model, the subsequent experiments were performed by evaluating the incorporation of [^{3}H]TdR in SH-SY5Y cells exposed for 24 h to the media in which A1 had been cultured for one day (A1-conditioned medium; A1-CM)(see Materials and Methods). As shown in Figure 2 (panel a), a 24-hour treatment of SH-SY5Y cells with the fresh A1-CM, used immediately after collection, surprisingly did not induce any modification of the incorporation of [^{3}H]TdR.

The effects, at much longer time intervals (3, 6 and 9 days), of the same A1-CM on the viability of neuroblastoma cells were subsequently evaluated. It has been first observed that control SH-SY5Y neuroblastoma cells grow up to 6 days after the transfer to a chemically-defined medium; afterwards the number of viable cells decreases to reach at the longest time of observation (9 days) a value significantly lower than that found at 6 days and close to that measured at the beginning of the experiment (time 0; Fig. 2, panel b). The visual inspection of the cultures showed that this effect was linked to a morphological differentiation of SH-SY5Y cells, which is then followed by cell death (data not shown) probably due to the absence, in the chemical medium, of trophic factors which support the growth of SH-SY5Y cells. When the

neuroblastoma cells have been incubated in freshly collected A1-CM, a significant greater number of viable SH-SY5Y cells was observed at any time interval when compared to control cells; this effect, already apparent at the 3rd day of incubation, became much more evident at 6 and 9 days (Fig. 2, panel b). In preliminary experiments, we observed that A1-CM still shows its stimulatory activity when applied to SH-SY5Y cells in the presence of FBS (absorbance at 560nm after 9 days of treatment: Control, 0.21± 0.03; A1-CM, 0.47 ± 0.03, $P < 0.05$), indicating either the presence of growth stimulatory factors in the A1-CM, which differs from those present in FBS, or the possibility of addictive effects between the growth stimulatory principles present in A1-CM and in FBS.

Figure 3 shows that the effect of the A1-CM was dependent on its concentration in the culture medium. No morphological differentiation of SH-SY5Y cells treated with A1-CM was observed at 3, 6 and 9 days of observation.

In order to verify the physico-chemical stability of the factor(s) responsible for the effects exerted by A1-CM, this CM was heated at 70°C for 20 min, a treatment known to induce denaturation of protein secondary structures.

The results have shown that this treatment did not decrease the activity of A1-CM on SH-SY5Y cell viability; a curve of growth similar to the one represented in Figure 2b was actually obtained (data not shown). However, a significant inhibitory effect on [3H]TdR incorporation, similar to that observed in co-culture experiments (see Fig. 1), was found using heat-denatured A1-CM (Fig. 4). Serendipitously, similar effects on both cell viability and [3H]TdR incorporation have been obtained using A1-CM stored at -20°C for a prolonged time (30 days) (Fig. 4).

On the basis of these results, it was decided to use, in all further experiments, conditioned media which had been stored for at least 30 days at -20°C.

It was therefore verified whether the effects of the CM on neuroblastoma cells were specific for A1, or whether other glial elements (i.e., type 2 astrocytes) might also induce it. SH-SY5Y cells were incubated with media conditioned for 24 hours by cultures enriched in A1 or in type 2 astrocytes. The results obtained show once again that the CM obtained from A1 induces a significant decrease of [^{3}H]TdR incorporation at 24 hours, followed by a significant increase of the number of viable cells after 6 days of treatment (Table 1). On the contrary, no significant effect on either [^{3}H]TdR incorporation or cell viability was observed using the CM obtained from type 2 astrocytes (Table 1).

On the basis of the observation that the decrease of the incorporation of [3H]TdR in SH-SY5Y cells treated with A1-CM is followed by an increase of the number of living neuroblastoma cells, it was decided to perform a time-course experiments in which the incorporation of [^{3}H]TdR in SH-SY5Y cells was evaluated using A1-CM submitted to 30 days of freezing, rather than the previously shown co-culture method (see Fig. 1).

In agreement with the results reported in Figure 1, it was found that the incorporation of [^{3}H]TdR in SH-SY5Y cells treated with undiluted A1-CM is characterised by an inhibitory phase evident at 12 hours and which lasts up to 24 hours (Table 2); this is followed by a progressive normalisation of this parameter, which returns to control levels 48 hours after the beginning of the incubation.

At this point, it was felt necessary to attempt at least an initial identification of the factor(s) involved in the opposite effects (inhibitory on the DNA synthesis and stimulatory on the cell survival/proliferation) exerted by the A1-CM on neuroblastoma cells. The physico-chemical characteristics (e.g., activation by heating and by freezing) of the factor(s) contained in A1-CM and responsible of the inhibitory effects, suggested a possible similarity with some components of the TGFß superfamily. In fact, TGFß, which may be also synthesised by astrocytes <Constam, 1992 #14>, is secreted in an inactive form complexed to a connecting glycopeptide which may be removed by physical (freezing or boiling) or chemical (extreme pH values) treatments, bringing to the free active form of the growth factor (Barnard et al., 1990). Consequently, a set of experiments was performed to verify the possible participation of TGFß in the inhibitory effects described above. Experiments of immunoneutralisation of the frozen A1-CM were performed using a pan-specific antibody, which recognises the major forms of TGFßs (see Materials and Methods), since different isoforms of TGFß have been so far characterised.

It has been found that the presence of the antibody totally blocks the inhibitory effect of A1-CM on [^{3}H]TdR incorporation (Figure 5, panel a) observed in SH-SY5Y cells after a 24-hour exposure; however, the same antibody was unable to modify the stimulatory effect of A1-CM on SH-SY5Y cell number (Figure 5, panel b).

DISCUSSION

The results reported in the present paper show that confluent cultures of rat A1 secrete some humoral factor(s) which are(is) able to stimulate the survival/proliferation of SH-SY5Y neuroblastoma cells, and that this effect is preceded by a transient decrease of DNA synthesis, detected measuring [^{3}H]TdR incorporation. Both effects are evident when neuroblastoma cells are maintained in co-culture with A1, or are exposed to the conditioned media obtained from cultures enriched in A1 (A1-CM) provided this was "activated" by either heating or freezing. However, the inhibitory effect on [^{3}H]TdR incorporation appears at short times of observation (24 hours) since a normalisation of this parameter is observed at 48 hours; as expected the stimulatory effect on cell number is evident only at longer time intervals (2 to 9 days). The observation of an increase of SH-SY5Y cell number, in presence of control values of [^{3}H]TdR incorporation, after 48 hours of co-culture, may be indicative of an effect of A1-released factors on cell duplication and/or on cell survival .

The rapid inhibitory effect on [^{3}H]TdR incorporation in neuroblastoma cells exerted by A1 is specific and not ascribable to a general toxic effect, since this effect was not evident when fresh A1-CM or the conditioned media derived from type 2 astrocytes have been used. These observations suggest that the decrease of [^{3}H]TdR incorporation is not due to the presence, in the conditioned medium, of molecules (e.g., thymidine derivatives or metabolites) which would interfere with the uptake, rather than with DNA incorporation of [^{3}H]TdR (Wilson, 1989).

The hypothesis that the inhibitory effect exerted by A1-CM on DNA synthesis might be due to a TGFß-like molecule was proposed on the basis that elements of the TGFβ family become fully active after heating or freezing; this (Barnard et al., 1990) was confirmed by the observation that the inhibitory effect was abolished by the presence of an anti-TGFß antibody. It is known that rat and mouse astrocytes release in culture various isoforms of TGFß in a latent form (Saad et al., 1991; Constam et al., 1992); however, the wide spectrum of the antibody used do not allow to identify which TGFß isoform (i. e., TGFß1, ß2, ß3 etc.) is directly responsible for the effects observed. The similarity of the results obtained from co-culture experiments and from those performed using A1-CM lead to believe that the same principle is involved; however, the observation that the medium of the co-culture is effective as such, while the A1-CM needs to be activated by heating or freezing, deserves some comment. It has been found that in heterotypic cell cultures TGFß may be present in free active form (Antonelli-Olridge et al., 1989; Sato and Rifkin, 1989); this is easily explained by the fact that the release of TGFß-activating enzymes from a cell population may be influenced by the other cell line present in the co-culture.

On the other hand, the data here presented also show that A1 secrete one or more factor(s) able to induce a strong stimulatory effects on survival and/or proliferation of neuroblastoma cells. In fact, the results obtained from co-culture experiments clearly show that when

neuroblastoma cells are in humoral contact with A1, they receive a trophic stimulus. At variance with the inhibitory effect on [^{3}H]TdR incorporation, this trophic effect becomes evident only after prolonged times of exposure of SH-SY5Y cells to cultures of A1 (2 days), or to their conditioned media (3, 6 and 9 days). The factor(s) involved in the stimulatory effects are highly active and their effects are still clearly and significantly evident at high dilution. This stimulatory effect of A1-CM did not require activation by freezing or heating, and was resistant to heat.

The results obtained from the analysis of the time course of [^{3}H]TdR incorporation show that the A1-CM-induced increase of the number of viable cells is not paralleled by an increase of thymidine uptake at the shorter time, this may be suggestive of an action of glial factor(s) on the survival rather than on the proliferation rate of SH-SY5Y cells. However, in a parallel set of experiments, it has been shown that the A1-CM is also able to increase the number of SH-SY5Y cells during their growth in FBS-supplemented medium, suggesting an addictive effect of the astrocytic factor(s) on cell proliferation.

A first obvious explanation of this effect include the possibility that the same TGFß, able to induce an early depression of DNA synthesis, may exerts some stimulatory effect at longer times. In fact, even if TGFßs appear to exert a general inhibitory effects on cell growth, stimulatory actions of this factor have been also reported; these are probably indirect, and mediated by the induction of other growth factors by the TGFß target cells (Barnard et al., 1990). However, the data here reported show that the neurotrophic effect exerted by A1-CM was not eliminated by the presence of the antibody directed to TGFßs, suggesting that the stimulatory effect is not due to this growth factor.

On the other hand, a linkage with the presence of TGFß might be foreseen, since mannose 6-phosphate, which is contained in the glycopeptide included in the latent form of TGFß, is known to exert a strong proliferative action on SH-SY5Y cells through the interaction with the IGF-II receptor (Feldman and Randolph, 1991); work is presently in progress to verify this hypothesis.

It is well known that glial cells synthesise and release neurotrophins, as well as a series of well characterised growth factors, cytokines and adhesion factors (Muller et al., 1995). In addition, a series of glia-derived factors active on neuroblastoma cells has been reported. These have been named gliostatin (Ueki et al., 1993), Glial Maturating Factor (GMF)(Lim et al., 1987; Lim et al., 1989; Lim et al., 1990), pleiotrophin (Wanaka et al., 1993) and human-Neuroblastoma Growth Inhibitory Factor (Eksioglu et al., 1994). In general, they exert an inhibitory effect on neuroblastoma cell proliferation which is usually linked to an induction of morphological differentiation (Monard et al., 1973). In the present paper no morphological differentiation of SH-SY5Y has been observed; a result similar to that obtained by Hirose and co-workers (Hirose et al., 1994)> who have found that the treatment of SH-SY5Y cells for 3 days with embryonic astrocytes CM did not produce any morphological differentiation of the

cells. The explanation of this apparent discrepancies on the effects of glial factors on morphological changes may be found in the work of Shea et al. (Shea et al., 1994); these authors have found that the induction of neuritogenesis of neuroblastoma cells by glial CM is dependent on the density and the age of the glial cultures, the maximal activity being observed in younger and non-confluent cultures. As previously mentioned, in the present study the CM was collected from confluent cultures of A1 maintained in serum free medium and therefore from cells with a pattern of secretory products which may be different from that of more dispersed glial cells. The results here reported have been obtained in experimental conditions that are different from those used in other studies quoted above; however, the observation that brain metastases of abdominal neuroblastoma effectively occur, suggests that the protocols here adopted may be the nearest to the "in vivo" pathological conditions.

It may be of interest to quote here the finding of humoral factors derived from glial cells (new-born rat cortical astrocytes and C6 rat glioma cells) which protect cultured mouse neuroblastoma cells against glutamate toxicity (Amano et al., 1994), as well as GDNF (Glia Derived Neurotrophic Factor), a member of the TGFß superfamily, which has been implicated in the survival of midbrain dopaminergic neurons and motoneurons "in vivo" and "in vitro"(Lin et al., 1993). More recently, it has also been reported that rat C6 glioma cells release factors which may increase the survival of SH-SY5Y cells in serum-deprived medium (Zuo and Yu, 1995). However, the different methodological approaches adopted (i.e., tumoral vs. normal astrocytes) and the different biochemical characteristics (i.e., heat sensitivity) of the factors involved in such effects, make very difficult a direct comparison of the results of the present study with those reported in the studies cited above, at least until the factors involved will not be characterised.

In conclusion, the results of the present study, even if they are limited only to the cell models adopted, indicate that trophic factors released by astrocytes may exert mainly a stimulatory effect on human neuroblastoma cell growth " in vitro"; these factor(s) may therefore exert either a permissive (by increasing survival) or a trophic (by increasing proliferation) action. The imbalance of the effects of such factors, or the acquired resistance of tumour cells to some of them (Johnson et al., 1993), may play a key role in favouring the occurrence of neuroblastoma-derived brain metastases. The future identification of the factor(s) involved in the trophic effects exerted by A1 on neuroblastoma cells "in vitro" will open new perspectives for the understanding of the mechanisms supporting the formation of metastases of this tumour in the central nervous system.

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REFERENCES

Amano S, Ohashi M, Ishiko T & Hazama F 1994 Humoral factors derived from glial cells protect cultured neuroblastoma cells against glutamate toxicity. *Brain Res* **645** 347-350.

Antonelli-Olridge A, Saunders KB, Smith SR & D'Amore PA 1989 An activated form of transforming growth factor-ß is produced by cocultures of endothelial cells and pericytes. *Proc Natl Acad Sci* **86** 4544-4548.

Astigarra I, Lejarreta R, Navajas A, Fernandez-Teijero A, Imaz I & Bezanilla JL 1996 Secondary central nervous system metastases in children with neuroblastoma. *Med Pediatr Oncol* **27** 529-533.

Banker GA 1980 Trphic interactions between astroglial cells and hippocampal neurons in culture. *Science* **209** 809-810.

Barnard JA, Lyons RM & Moses HL 1990 The cell biology of transforming growth factor beta. *Biochim Biophys Acta* **1032** 79-87.

Biedler J L, Helson L & Spengler B A 1973 Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Res* **33** 2643-2652.

Bouffet E, Doumi N, Thiesse P, Mottolese C, Jouvet A, Lacroze M, Carrie C, Frappaz D & Brunat-Mentigny M 1997 Brain metastases in children with solid tumors. *Cancer* **79** 403-410.

Constam DB, Philipp J, Malipiero UV, Dijke P, Schachner M & Fontana A 1992 Differential expression of trasforming growth factor-β1,-β2,and β3 by glioblastoma cells, astrocytes, and microglia. *J Immunol* **148** 1404-1410.

De Lean A, Munson PJ & Rodbard D 1978 Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and phisiological dose-response curves. *Am J Physiol* **235** E97-E102.

Eksioglu YZ, Iida J, Asai K, Ueki T, Nakanishi K, Isobe I, Yamagata K & Kato T 1994 Human neuroblastoma growth inhibitory
factor (h-NGIF) derived from human astrocytoma conditioned medium has neurotrophic properties. *Brain Res* **644** 282-290.

Feldman E L & Randolph A E 1991 Mannose 6-phosphate potentiates insulin-like growth factor II effects in cultured human neuroblastoma cells. *Brain Res* **562** 111-116.

Giulian D, Vaca K & Johnson B 1988 Secreted peptides as regulators of neuron-glia and glia-glia interactions in the developing nervous system. *J Neurosci Res* **21** 487-500.

Hatten ME & Mason CA 1990 Mechanisms of glial-guided neuronal migration in vitro and in vivo. *Experientia* **46** 907-916.

Hirose Y, Imai Y, Nakajima K, Takemoto N, Toya S & Kohsaka S 1994 Glial conditioned medium alters the expression of amyloid precursor protein in SH-SY5Y neuroblastoma cells. *Biochem Biophys Res Commun* **198** 504-509.

Johnson MD, Jennings MT, Gold LI & Moses HL 1993 Transforming growth factor-ß in neural embryogenesis and neoplasia. *Hum Pathol* **24** 457-462.

Kato T, Sakazaki Y, Yamakawa Y, Kato H, Naganawa N, Kato T, Tsunooka H, Masaoka A & Tanaka R 1983 Inhibition of growth of mouse neuroblastoma cells by protein factor derived from rat glioblasts. *Dev Brain Res* **3** 645-651.

Lim R, Hicklin D J, Ryken T C & Miller J F 1987 Endogenous immunoreactive glia maturation factor-like molecule in astrocytes and glioma cells. *Dev Brain Res* **33** 49-57.

Lim R, Liu Y & Zaheer A 1990 Glia maturation factor β regulates the growth of N18 neuroblastoma cells. *Dev Biol* **137** 444-450.

Lim R, Miller J F & Zaheer A 1989 Purification and characterization of glia maturation factor β: a growth regulator for neurons and glia. *Proc Natl Acad Sci USA* **86** 3901-3905.

Lin L-FH, D D, Lile J, Bektesh S & Collins F 1993 GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260** 1130-1132.

Lindsay RM 1987 Adlt rat brain astrocytes support survival of both NGF-dependent and NGF-insensitive neurons. *Nature* **282** 80-82.

LoPachin RMJ & Aschner M 1993 Glial-neuronal interactions: relevance to neurotoxic mechanisms. *Toxicol Appl Pharmacol* **118** 141-158.

Lydaki E, Bolonaki I, Stiakaki E, Dii G & Kalmanti M 1997 Isolated intracranial metastasis of neuroblastoma 2 years after completion of therapy. *Acta Pediatr Japon* **39** 79-82.

Manthorpe M, Fagnani R, Skaper S D & Varon S 1986 An automated colorimetric microassay for neuronotrophic factors. *Dev Brain Res* **25** 191-198.

Melcangi R C, Celotti F, Castano P & Martini L 1993 Differential localization of the 5α–reductase and the 3α- hydroxysteroid dehydrogenase in neuronal and glial cultures. *Endocrinology* **132** 1252-1259.

Monard D, Solomon F, Rentsch M & Gysin R 1973 Glia-induced morphological differentiation cells. *Proc Natl Acad Sci USA* **70** 1894-1897.

Muller HW, Junghans U & Kappler J 1995 Astroglial neurotrophic and neurite-promoting factors. *Pharmac Ther* **65** 1-18.

Pahlman S, Mamaeva S, Meyerson G, Mattsson M E,K, Bjelfman Ortoft E & Hammerling U 1990 Human neuroblastoma cells in culture: a model for neuronal cell differentiation and function;. *Acta Physiol Scand Suppl*. **592** 25-37.

Saad B, Constam D B, Ortmann R, Moos M, Fontana A & Schachner M 1991 Astrocyte-derived TGF-b2 and NGF differentially regulate neural recognition molecule expression by cultured astrocytes. *J Cell Biol* **115** 473-484.

Sakazaki Y, Kato T, Kato H, Ito J, Tanaka R, Naganawa N, Kato T, Masaoka A & Tsunooka H 1983 Characterization and partial purification of neuroblastoma growth inhibitory factor from the culture medium of glioblasts. *Brain Res* **262** 125-135.

Sato Y & Rifkin DB 1989 Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-$ß_1$-like molecule by plasmin during co-culture. *J Cell Biol* **109** 309-315.

Shaw PJ & Eden T 1992 Neuroblastoma with itracraial involvemet: an ENSG Study. *Med Pediatr Oncol* **20** 149-155.

Shea TB, Beermann ML, Honda T & Nixon RA 1994 Secretion of amyloid precursor protein and laminin by cultured astrocytes is influenced by culture conditions. *Neurosci Res* **37** 197-207.

Tasdemiroglu E & Patchell RA 1997 Neurologic complications of the primary pediatric extracranial neuroblastomas. *Neurological Res* **19** 45-50.

Ueki TN K, Asai K, Okouchi Y, Isobe I, Eksioglu Y Z, Kato T & Kohno K 1993 Neurotrophic action of gliostatin on cocultured neurons with glial cells. *Brain Res* **622** 299-302.

Wanaka A, Carroll S L & Milbrandt J 1993 Developmentally regulated expression of pleiotrophin, a novel heparin binding growth factor, in the nervous system of the rat. *Dev Brain Res* **72** 133-144.

Weyl-Ben Arush M, Stein M, Perez-Nachum M, Dale J, Babilsky H, Zelnik N & Kuten A 1995 Neurologic complications in pediatric solid tumors. *Oncology* **52** 89-92.

Wilson AP 1989 Cytotoxicity and viability assays. In *Animal cell culture. A practical approach*, pp 183-216. Eds RI Freshney. Oxford: IRL Press.

Zuo D & Yu PH 1995 Increase of survival of dopamiergic neuroblastoma in co-cultures with C-6 glioma by R-(-)-deprenyl. *Progr Brain Res* **106** 199-205.

Table 1

Effect of conditioned media obtained from type 1 and from type 2 astrocytes on the incorporation of [^{3}H]TdR in SH-SY5Y cells (24-hour exposure) and on their viability (6-day exposure).

Glial cells	[^{3}H]TdR incorporation[a]	viability (MTT assay)[a]
Type 1 astrocytes	−40 ± 7[b]	+93 ± 3[b]
Type 2 astrocytes	+12 ± 5	−8 ± 1

(a) Values are expressed as mean percent variation ± SD respect to control cultures exposed to non-conditioned chemical-defined medium.
(b) Significant $P<0.05$ vs. respective controls.

Table 2

Time course of [3H]TdR incorporation in SH-SY5Y cells exposed to non-conditioned control medium and A1-CM

Time of treatment (hours)	Control[a]	A1-CM[a]	% inhibition
12	33420 ± 6456	16212 ± 1659[b]	51.5
18	44671 ± 1790	21288 ± 750[b]	52.3
24	46197 ± 3335	27472 ± 2841[b]	40.5
36	50342 ± 7214	37188 ± 1375[b]	26.1
48	56494 ± 1556	54807 ± 4307	2.9

(a) Values are expressed as mean of cpm/well ± SD; (b) Significant $P<0.05$ vs Control

Figure legends

Figure 1

Effects of co-culture between SH-SY5Y cells and type 1 astrocytes in chemical-defined medium on the [^{3}H]TdR uptake (a) and on the number (b) of SH-SY5Y. Type 1 astrocytes have been cultured on 0.45 μm membrane inserts and put in contact with the culture medium present in culture wells contained SH-SY5Y cells adherent on the plastic surface. In this system only the exchange of soluble signals is possible. The number of living cells was evaluated by an hemocytometer and the trypan blue-exclusion test.

The values represent the mean ± SD of 3 independent experiments run in triplicate.***** Significant $P<0.05$ vs control (C).

Figure 2

Incorporation of [^{3}H]TdR in SH-SY5Y cells treated with control non-conditioned chemical-defined medium and fresh A1-CM (a) . Viability of SH-SY5Y cells maintained for 3, 6 and 9 days in chemical-defined medium (close symbols) or in the A1-CM (open symbols) (b). The amount of viable cells has been evaluated by colorimetric MTT assay (see Materials and Methods).

Values represent mean ± SD. (* Significant $P < 0.05$ vs control).

Figure 3

Dose-related effect of different dilution of the A1-CM on the proliferation of SH-Y5Y cells (9-day exposure). The amount of viable cells has been evaluated by colorimetric MTT assay.

Values represent mean ± SD. (* Significant $P < 0.05$ vs control).

Figure 4

Incorporation of [^{3}H]TdR in SH-SY5Y cells exposed to non-conditioned chemical-defined medium, fresh A1-CM or A1-CM which has been submitted to heating (70°C/ 20 min) or prolonged freezing (-20°C/ 30 days).

Values represent mean ± SD. (* Significant $P < 0.05$ vs control).

Figure 5

Effects of the presence of an anti-TGFßs antibody (5 μg/ml) on SH-SY5Y cells exposed to A1-CM. (a) [^{3}H]TdR incorporation was measured in SH-SY5Y cells exposed for 24 hours to non-conditioned chemical-defined medium (control, C) or A1-CM. (b) Cell viability was evaluated by colorimetric MTT assay after a 3-day exposure to control medium or A1-CM. Values represent the mean ± SD. * Significant $P<0.05$ vs control (C).