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EFFECTS OF EPIDERMAL GROWTH FACTOR ON THE [3H]-THYMIDINE UPTAKE IN THE SK-N-SH AND SH-SY5Y HUMAN NEUROBLASTOMA CELL LINES

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ABSTRACT - The studies on the factors that regulate the biology of the neuroblastoma cell lines may offer important information on the development of tissues and organs that derive from the neural crest. In the present paper we study the action of epidermal growth factor (EGF) on two human neuroblastoma cell lines: SK-N-SH which is composed at least of two cellular phenotypes (neuroblastic and melanocytic/glial cells), and its pure neuroblastic subclone SH-SY5Y. The results show that EGF (10 ng/ml) significantly stimulates the incorporation of [3H]-thymidine in the SK-N-SH cells only in the presence of fetal bovine serum (FBS) (control = 58285 ± 9327 cpm; EGF =75523 ± 4457; $p<0.05$). Such effect is not observed in the presence of a chemical defined medium, that is, in the absence of FBS (control = 100997 ± 4375; EGF = 95268 ± 4683; NS). In the SH-SY5Y cells the EGF does not modify the incorporation of [3H]thymidine either in the presence of 10% of BFS (control = 113838 ± 6978; EGF = 119434 ± 9441; NS) or in its absence (control = 46197 ± 3335; EGF = 44472 ± 3493; NS). The results here reported suggest that: a) EGF may affect the proliferation of cells derived from a primary human neuroblastoma; b) this is evident by the EGF-induced increase of [3H]-thymidine incorporation in SK-N-SH cells; c) it is required the presence of other growth factors, present in the FBS, for the mitogenic action to be accomplished; d) since the pure neuroblastic SH-SY5Y cell line are refractory to the EGF, the effects observed in SK-N-SH cells probably occur on the melanocytic/glial cell subpopulation.

KEY WORDS: epidermal growth factor, cells cultured, neuroblastoma, growth factors.

Efeitos do fator de crescimento epidérmico sobre a captação de [3H]-timidina nas linhas celulares de neuroblastoma humano SK-N-SH e SH-SY5Y

RESUMO - Os estudos dos fatores que regulam a biologia das linhas celulares de neuroblastoma podem oferecer importantes informações sobre o desenvolvimento de tecidos e de orgãos que derivam da crista neural. No presente estudo analisamos a ação do fator de crescimento epidérmico (EGF) sobre duas linhas celulares de neuroblastoma humano: a linha SK-N-SH que é constituída de dois fenotipos celulares (neuroblástico e melanocítico/glial), e o seu subclone puro neuroblástico SH-SY5Y. Os resultados mostram que o EGF (10ng/ml) estimula significativamente a incorporação de [3H]-timidina na linha SK-N-SH somente na presença de 5% de soro fetal bovino (SFB) (controle = 58285 ± 9327 cpm; EGF = 75523 ± 4457; $p<0,05$). Tal efeito não é observado na presença de meio definido químico, isto é, na ausência de SFB (controle = 100997 ± 4375; EGF 95268 ± 4683; NS). Nas células SH-SY5Y o EGF não modificou a incorporação de [3H]-timidina nem na presença de 10% SFB (controle = 113838 ± 6978; EGF=119434 ± 9441; NS), nem na sua ausência (controle = 46197 ± 3335; EGF = 44472 ± 3493; NS). Os resultados aqui apresentados sugerem que: a) o EGF pode interferir na proliferação de células derivadas primariamente de um neuroblastoma humano; b) isso é evidente pelo aumento da incorporação de [3H] timidina pelas células SK-N-SH tratadas com EGF; c) é necessário a presença de outros fatores de crescimento, presentes no SFB, para que exerça esta ação mitógena; d) desde que a linha celular neuroblástica pura é refratária ao EGF, os efeitos descritos nas células SK-N-SH, provavelmente, ocorrem nas subpopulações de células melanocíticas/gliais.

PALAVRAS-CHAVES: fator de crescimento epidérmico, cultura de células, neuroblastoma, fatores de crescimento.

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An important matter in the study of embryology is to establish how primordial cells that arise from several parts of the neural crest can give origin to highly differentiated cells with very specific functions. During the migration, the cells that compose the neural crest react to the local environmental factors to turn themselves into specialized tissues typical of their final destination[38]. However, it is not clear enough yet what are the environmental factors involved and how they work on the various cells of the neural crest.

It has been described that glucocorticoids[12,13] and growth factors such as nerve growth factor (NGF)[12,13], fibroblast growth factor (FGF)[6] and insulin-like growth factors family (IGF)[22] may influence the development of the cells originated in the neural crest. It has also been shown that epidermal growth factor (EGF) is able to influence the action of NGF during the differentiation of the post-natal chromaffin cells in sympathetic neurons[38].

EGF is a peptide which shows a mitogenic action over a variety of epithelial and mesenchymal cells in culture[8,16]. A series of findings show that EGF, together with transforming growth factor-alpha (TGF-alpha), which belongs to the same family of peptides and works through the same receptor (EGF-R), operates over the nervous tissue as a trophic and mitogenic factor. EGF, TGF-alpha and their mRNAs may be identified in many parts of the brain. It has been suggested that TGF-alpha is the main member in this family of growth factors active during the fetal life (for discussion and reference Fisher and Lakshmanan[16]). The brain of newborn rats show higher levels of EGF and EGF-R than adult rats (review[16]); these observations suggest that the EGF system activity decreases as the maturation of the nervous tissue progresses. EGF and TGF-alpha also support the neuronal survival and stimulate the proliferation of the astrocytes (for reference Cestelli et al.[10], Fischer and Lakshmanan[16], Ma et al.[25]). Of special interest is the ability of TGF-alpha and EGF to stimulate "in vitro" the release of hyaluronic acid and chondrointin sulfate from the quails neural crest cells[15] and to induce the development of the neuroepithelial progenitor cells of the rat retina[1]; these data suggest that the two growth factors may play an important role during the early phases of the development of the nervous system.

In more complex organisms, where the study of the mutations is extremely difficult or impossible, the relative contributions of the intrinsic or environmental factors that define the fate of the cells forming the neural crest, may be studied by "in vitro" systems and under controlled conditions. For instance, studies were perfomed using purified populations of cells obtained from the neural crest[38]. Another possible approach to this sort of studies is to use tumor cell lines, characterized by cells with morphological and biochemical features similar to those of the embryonic cells[28,32,35,36]. The advantage of the use of tumor cell lines consists in the reproducibility of the results, the long term survival of the cells in culture, and consequently the possibility of long term studies. On the other hand, there is the possibility of acquiring a better knowledge of the mechanisms involved in the phenotypic tumoral change with the associated clinical consequences[11].

In this study we used two human neuroblastoma cellular lines: the SK-N-SH line, composed of neuroblastic and melanocytic/glial cell types and its subclone SH-SY5Y, composed mainly of the neuroblastic-type cells. SK-N-SH cells line was obtained from a 4-year-old girl with bone marrow neuroblastoma metastasis[3]. This cell line comprises two distinctly different cell types[3-5,9,37]. One is a small spine cell with high levels of dopamine-beta-hydroxylase activity, an enzyme distributed only in sympathetic nervous tissues. This type resembles a primitive sympathoblast. The other type of cell does not express activities for the catecholamine-biosynthetic enzymes and could denote either melanocyte or neurilemmal (Schwann) cells. Both cell types can undergo bidirectional coordinate morphological and biochemical interconversion in culture[4,5,9,36,37].

SK-N-SH line presents many of the neural crest cells properties in culture. From the SK-N-SH line the SH-SY5 line was cloned, and from the SH-SY5 line the SH-SY5Y was cloned[36,37]. The latter cell line presents a well defined neuroblast phenotype and has the two primary enzymes involved

in the synthesis of the norepinephrine, tyrosine hydroxylase and dopamine beta-hydroxylase[36,37]. SH-SY5Y cell has muscarinic[21], opioid[43], NGF[34] and EGF receptor[23,27]. SH-SY5Y cell is morphologically and biochemically similar to the human fetal sympathetic ganglion cell[32]. Both human neuroblastoma cell lines (SK-N-SH and SH-SY5Y) characteristics become, therefore, of a valuable significance for the study of the endogenous and exogenous influences of the tissues proceeding from the neural crest.

The studies have been made in order to verify the effect of EGF, a growth factor widely involved in the neural embryogenic process, on the proliferation of the neuroblastoma cells, since little is known about the mechanism through which the EGF influence neural crest cells development and neuroblastoma growth.

MATERIALS AND METHODS

Chemicals

[Methyl- 3H]-thymidine (25 ci/mmol) was obtained from Amersham (Amity, Milan, Italy). The culture media MEM (Minimal Essential Medium) and DMEM (Dulbecco's Modified Eagle's Medium), non-essential amino-acids, sodium pyruvate, glutamine and antibiotics were supplied by Biochrom K.G., (Berlin, Germany). The fetal bovine serum (FBS) was purchased from GIBCO - USA. Trypsin-EDTA and EGF were supplied by Sigma (St. Louis, MO, USA).

Cells and cell cultures

SK-N-SH and SH-SY5Y human neuroblastoma cells were obtained from Dr. June Biedler (Memorial Sloan-Kettering Cancer Center). These cells were cultured in MEM containing 5% FBS (SK-N-SH) or 10% FBS (SH-SY5Y), non-essential amino-acids, sodium pyruvate and antibiotics (100 IU/ml penicillin G sodium; 100 g/ml streptomycin sulfate). Culture media was changed every 72 h. In some experiments, the cells were maintained in chemical defined medium made with DMEM containing 5 mg/ml bovine insulin; 100 mg/ml human transferrin; 20 nM progesterone; 0.01 nM estradiol; 100 mM putrescin dihydrochlorydrate and 30 nM selenite sodium.

The cells were grown at 37°C, in 5% CO2/95% air atmosphere. Cell viability was determined by the trypan blue exclusion test.

[3H]-thymidine incorporation studies[14]

SK-N-SH and SH-SY5Y neuroblastoma cells were plated in 24-well plates (Linbro-Flow Laboratories SA, Ayrshire, UK) at a density of 50,000 cells/well in 1 ml of MEM additioned with BFS. Three days after seeding, the medium was replaced by MEM without FBS. Forty-eight hours later, the cell cultures were refed with complete or chemical defined medium containing the EGF. After a further 20 hour incubation, [3H]-thymidine (10 μCi/ml) was added to the media and the samples were incubated for an additional period of 4 hours. Cells were then washed with 0.5 ml/well of warm phosphate-buffered saline (PBS) and then replaced with 1 ml/well of cold 10% trichloroacetic acid. The cell monolayer was then lysed by a treatment with 200 μl NaOH (6 N) for 10 minutes. The cell lysate was mixed with 7 ml of Instagel scintillation cocktail (Packard, Milan, Italy) and the radioactivity counted with an efficiency of 60%. Three wells were used for each treatment.

Statistical analysis

Data from the experiments were evaluated according to the Dunnett's test after one-way analysis of variance. Results are expressed as mean ± standard deviation (M±SD).

RESULTS

The results obtained show that EGF (10 ng/ml) significantly stimulates [3H]-thymidine uptake in the SK-N-SH cells ($p<0.05$) in the presence of 5% FBS (Fig 1A). Such effect did not occur when the experiment was made in serum-free chemical defined medium (Fig 1B).

In the SH-SY5Y cell line, the same dose of EGF did not modify [3H]-thymidine uptake in both the conditions under study: with either FBS or chemical defined medium (Table 1). Even

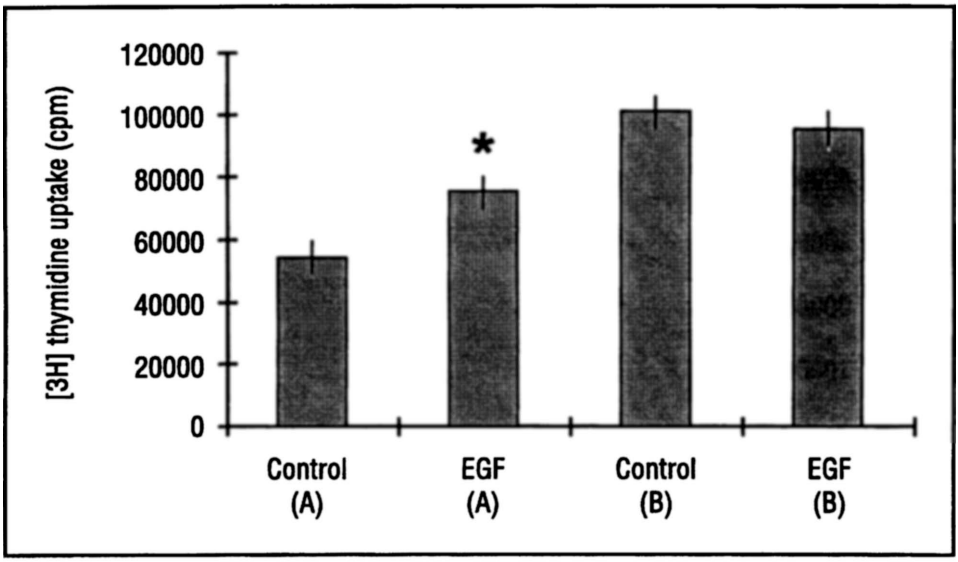

Fig 1. EGF (10 ng/ml) stimulative effect in the [3H]-thymidine uptake on the SK-N-SH cells with 5% FBS in the culture medium (A); absence of effect without FBS in culture medium (chemical defined medium) (B). All other details are described under "Results" and "Material and Methods". Values are means ± SD.
** $p < 0.05$ vs control.*

Table 1. Lack of effect in the [3H]-thymidine incorporation on the SH-SY5Y cells with various concentrations of EGF, in the presence of DMEM medium with 10% FBS or chemical defined medium. All other details are described under "Results", and "Material and Methods". Values are means ± DS.

EGF (ng/ml)	FBS 10% (cpm/well)	Chemical defined medium (cpm/well)
Control	113838 ± 6978	46197 ± 3335
1	115082 ± 9813	–
10	119434 ± 9441	44472 ± 3493
100	116037 ± 3793	–

smaller (1 ng/ml) or larger (100 ng/ml) doses of EGF were not able to significant by affect the [3H]-thymidine incorporation in these cells (Table 1). On the basis of the negative results obtained on the [3H]-thymidine uptake in SH-SY5Y cells, we examined the possibility that the EGF effect had occurred before or after the observation time (24 h) chosen for the experiment perfomed on cells cultured in the presence of FBS, since the presence of chemical medium may influence on the cellular cycle of the neuroblastoma cells. As shown in Figure 2, EGF treatment (10 ng/ml) did not modify the [3H]-thymidine incorporation in SH-SY5Y cells after incubation intervals ranging from 12 to 46 hours.

DISCUSSION

The present results show that EGF is able to increase [3H]thymidine incorporation, i.e. DNA synthesis, in human neuroblastoma cells. This EGF stimulatory effect over [3H]-thymidine incorporation in the SK-N-SH cell line occurred when the experiment was conducted in the presence of 5% FBS, whereas such effect was not present when the same experiment was conducted with a

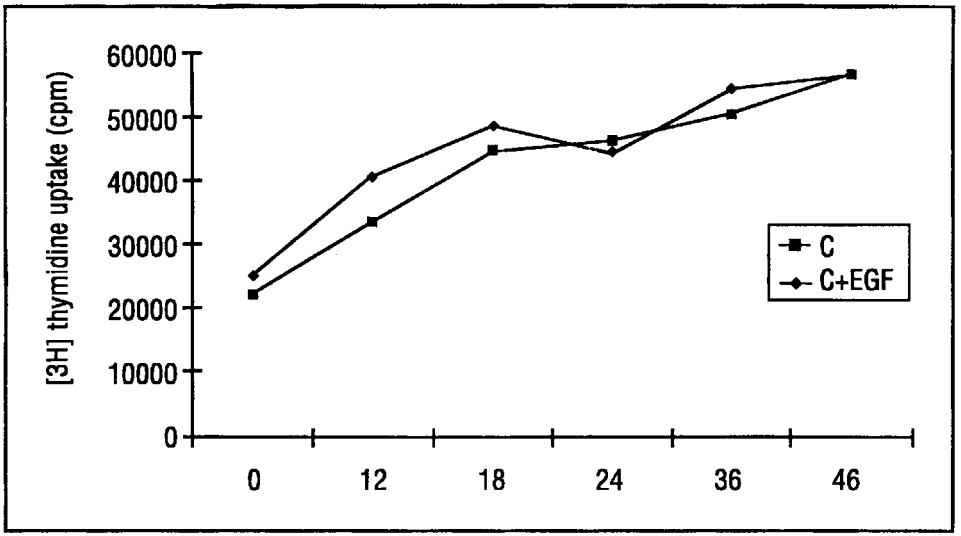

Fig 2. Absence of effect of EGF (10 ng/ml) in the [3H]-thymidine uptake on the SH-SY5Y cells, with chemical defined medium, during 46 h of observation. The cells were placed on a multiwell of 24 and arrest in phase G0/G1 of the cellular cycle as described under "Results", and "Material and Methods". At the time 0 the cells in every three wells were treated with either chemical medium (C) or 10 ng/dl EGF (C + EGF). Four hours before the indicated time [3H]-thymedine was added.

chemical defined medium, that is, without FBS. These findings suggest that the EGF needs other growth factors present in the FBS to exert its effects. The survival and the differentiation of a cell population depends on the synergistic activity of trophic factors that frequently work in parallel (see Cestelli et al.[10]). In order to explain such action of growth factors, it was proposed a competency/progression model, in which a growth factor induce a stage of cell competence for a specific action of another factor. For instance, PDGF sensitizes the 3T3 BALB/C fibroblasts to a mitogenic response generated by EGF or by the IGF-I (see Goustin et al.[17]). IGF-II promoted SH-EP growth only in the presence of low concentrations of calf serum or EGF[23]. A similar phenomenon was also described for the immortalized sympathoadrenal progenitor cells. Undifferentiated sympathoadrenal cells do not express NGF receptors, and therefore do not replicate and do not depend on the NGF for their survival. However, the treatment of such cells with FGF allows the appearance of NGF-R and induces a dependence from the NGF[6].

On the other hand, it has also been reported that EGF may exert its effects on cell proliferation through the activation of other growth factor systems. For example, in the chromaffin cells of the newborn rat, EGF may induce a dependence on NGF during the neuronal differentiation of the sympathetic system[38]; in the brain, EGF and FGF-alpha present synergistic effects over the maturation and over the functionality of such cells[16]. The FBS contains many growth factors and among them PDGF is the most represented (see Goustin et al.[17]). Actually, a variety of neural crest-derived tumor cell lines, including the SK-N-SH neuroblastoma cells have been reported to express PDGF receptors and to have mitogenic activity in neuronal cells[31]. It has been shown a synergism between EGF and PDGF in thecal ovary cells in culture[26]. These findings lead us to speculate that PDGF present in serum may be responsible for the EGF action in the SK-N-SH only in the presence of FBS.

Several other explanations are possible to justify the lack of effect of EGF in neuroblastoma cells growing in chemical defined medium. This culture medium contains high levels of insulin which might interfere with the EGF effect on the target cell. On the basis of the data present in the literature, it has been possible to postulate that insulin may have the following actions: 1) A direct

inhibitory effect on the EGF binding SK-N-SH cells; such fact has been shown in Swiss 3T3 cells in which insulin and IGF-I decrease the EGF binding to its own receptors[7]. 2) An indirect inhibitory effect mediated by another growth factor produced by the target cells over the insulin effect and that, on its turn, would cause a decrease of the EGF-R; for example, NGF decreases the number of EGF-R on the pheochromocytoma PC12 cells[7,19]. SK-N-SH cells are among the few neuroblastoma lines that have NGF receptors[2]. It may be possible that insulin stimulates the secretion of a growth factor from the cells that compose the SK-N-SH clone and that this factor, in turn, suppresses the EGF's effect on these cells. 3) A differentiating effect over the SK-N-SH cells; for that reason they loose the ability to respond to the mitogenic activity of EGF. For example, insulin and IGF-I cause the differentiation of human neuroblastoma cells which in turn no longer respond to EGF[32].

We did not find any kind of response to EGF from the SH-SY5Y cell line, a subclone with a neuronal phenotype derived from SK-N-SH cells and this might be due to the larger neuronal-like differentiation of the subclone[36,37]. Many experiments have shown that EGF does not exert a major influence on the neuronal cells growth. For instance, it has been reported that mouse N1E-115 neuroblastoma cells loose this ability to bind to EGF after the beginning of this neuronal differentiation. These cells, after a few days in a serum-free system, turn into a highly differentiated cell phenotype, loosing its ability to grow in response to the EGF stimulus[29]. Similarly, EGF does not influence the proliferation of IMR-32 cells[33], which is considered a pure neuroblastoma cell line[41]. Furthermore EGF does nor interfere with the survival of neuronal component in primary cultures of rat fetal encephalic septal cells[20]. In fact, neurons show a very weak ability to bind and respond to EGF, when compared to other cellular types[39,40].

The effects of EGF can therefore occur mostly on the melanocytic/glial component present only in the SK-N-SH mixed cell line. The EGF is, in fact, acknowledged as a growth factor with activity mainly over the non-neuronal type of cells[8,17], and it has been reported to stimulate the [3H]-thimidine incorporation[24] and the growth of the astroglial cells[20,42]. The data herein analyzed suggest that in the SK-N-SH neuroblastoma mixed cellular line EGF favors the growth of a class cell non neuronal.

We may also suppose that the neuronal-like differentiation of cells causes a loss of the ability to respond to EGF proliferating stimulus, whereas the cells that develop in the non neuronal sense retain this ability unchanged. Many experiments support this hypothesis. For instance, it has been shown that the SH-EP cell line, a subclone derived from the SK-N-SH cells with melanocytic characteristics, present much more EGF-R than the SH-SY5Y neuroblastic subclone[23,27]. On the other hand, the PC12 cells present the EGF-R[19] and respond to the EGF proliferating stimulus[30]. In these cells, NGF causes a neuronal differentiation which may be associated to a loss of EGF-R[7]. These data inter-relate a gradual change versus the neuronal differentiation with a decrease in the ability to bind to respond to EGF. These data also suggest that the cells that differentiate may change the characteristics of their receptors to growth factors and hormones according to the amount of receptors present on the membrane[7]. It is therefore possible to postulate that the differentiation of the sympathoadrenal progenitor cells in neuroblasts induces a decrease in the number of EGF-R and/or their ability to respond to the proliferating stimulus of EGF.

We can distinguish two kinds of tumors that may originate from neuroblasts: the neuroblastoma, consisting of non-differentiated neuroblastic cells, which behaves as a malignant tumor, and the ganglioneuroma, originating from the mature ganglions, with a benign behavior[18]. Frequently neuroblastoma show spontaneous regression due to differentiation into benign ganglioneuromas (see Pahlman et al.[32]). An attractive line of research would be that examining the hypothesis that the sensitivity loss to EGF may have a role in this tumoral transformation.

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