

Morphometric characteristics of TGF- β 1-positive cells of fetal rat brain *in vitro*



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ABSTRACT

One of the directions of cell therapy being developed for brain gliomas is the use of the neurogenic stem and progenitor cells (NSCs/NPCs). There are data on the anti-tumor and immunomodulating properties of the NSCs/NPCs the mechanisms of which were not disclosed yet. One of the potential targets for tumor therapy is the transforming growth factor β (TGF- β 1) which is thought to be one of the key molecules in the regulation of proliferation, differentiation and cell survival or apoptosis. In the view of available information about the possibility of TGF- β 1 production by the mammalian multipotent NSCs/NPCs, the aim of this work was to study the TGF- β 1-positive cells in the dynamics of cultivation of fetal brain neurogenic cells as a potential source of anti-tumor or immunomodulating effects of these cells.

MATERIAL AND METHODS. The fetal rat brain cells on 14th (E14) day of gestation were used as the source for cultivation in standard conditions (DMEM + 1 % fetal bovine serum) and studied on the 2nd and 37th day by morphometry and immunocytochemistry.

RESULTS. In the fetal rat brain cell cultures, the TGF- β 1-positive cells made 22.04 ± 2.33 % and the nestin-positive cells made 49.16 ± 10.60 % of the total cells number. The morphometric parameters of TGF- β 1-positive cells exceeded the corresponding values of negative cells (average values of cross-sectional areas of the cytoplasm, cross-sectional areas of the nucleus, nuclear-cytoplasmic ratio). During cultivation the relative amount of TGF- β 1-positive cells was slightly decreased (15.27 ± 9.80 %, $p = 0.7$) and their sizes were increased. On the 37th day of cultivation the sizes of TGF- β 1-positive cells and their nuclei were smaller in the comparison with the TGF- β 1-negative cells.

CONCLUSIONS. The presence of TGF- β 1 expression by part of neurogenic cells of fetal rat brain (E14) *in vitro* was found, which persisted throughout cultivation (~5 weeks). Significant quantitative differences of morphometric parameters of TGF- β 1-positive and negative cells were detected.

KEYWORDS: fetal rat brain cell culture; TGF- β 1; nestin; morphometry; immunocytochemistry

One of the directions of cell therapy being developed for brain gliomas is the use of neurogenic stem and progenitor cells (NSCs/NPCs) [1-4]. It was showed that NSCs and NPCs can migrate to the glioblastomas inhibiting glioma growth in the mice and rats and extend the lifespan of tumor-bearing animals [5-7] owing to induction of long-term anti-tumor response [5, 8]. Cellular and molecular similarity of the NSCs and brain tumor stem cells was detected [9, 10]. Nevertheless, the mechanisms of anti-tumor and immunomodulating properties of NSCs/NPCs remain unknown so far.

Currently, cell transplantology is used in the treatment of a wide range of diseases, including neuro-oncological ones. The transforming growth factor β (TGF- β) and its related cellular signal pathways are considered as one of the potential targets for anti-tumor therapy [11-16]. TGF- β is thought to be one of the key factors of the regulation of

proliferation, differentiation and cell survival or apoptosis [14, 15, 17]. Under the physiological conditions, TGF- β plays an important role in the processes of embryo- and morphogenesis and in the maintenance of tissue homeostasis [13, 18].

In the pathological conditions, brain glial tumors in particular, TGF- β can play a dualistic role. On the one side, this factor is an important mediator of the malignant phenotype of human brain gliomas: it modulates invasiveness, angiogenesis, immune control escape and maintenance of the stem cells of the brain tumors [16, 19-21]. On the other side, TGF- β is viewed as a tumor-inhibiting factor since it is a powerful inhibitor of proliferation of various types of cells [16, 22].

It was showed that TGF- β is a highly pleiotropic cytokine playing a key regulatory role in many aspects of the immune response: it directly inhibits cytotoxic activity of the natural killers, macrophages and CD8⁺ cytotoxic

lymphocytes (CTL); it can also inhibit activation and expansion of tumor-specific populations of T-helpers and CTL and enhance generation of immunosuppressive T-regulatory cells [11, 23].

There are now definite data regarding TGF- β 1 expression by the fetal brain cells. Thus Pelton R. et al. in their immunohistochemical investigation of the brain slices observed a high expression of TGF- β 2, TGF- β 3 and a low expression of TGF- β 1 in the embryonic CNS tissue of the mouse during a period from 12.5th to 18.5th dpc [18]. Several other investigators showed that multipotent NPCs of humans, rats and mice are capable to produce all TGF- β isotypes, and TGF- β 1, TGF- β 2 in particular [24-26].

Since TGF- β 1-expressing neurogenic cells are the potential source of anti-tumor or immunomodulating effects, of their possible use in therapy of brain gliomas it seems to be the capacities of NSCs/NPCs for *in vitro* TGF- β 1 expression.

Therefore this work aimed to investigate the TGF- β 1-positive cells in the dynamic of culture of the fetal brain neurogenic cells.

MATERIALS AND METHODS

All investigations with the use of experimental animals are carried out in keeping with the Law of Ukraine «On protection of animals from cruelty», The European Convention for the Protection of vertebrate animals used for experimental and other scientific purposes (European convention, Strasburg, 1986) with a full consideration of the bioethical principles and norms of the biological safety and approved by the Bioethical Committee of the State Institution «Romodanov Neurosurgery Institute, NAMS of Ukraine». Six white outbred female rats weighing 200 ± 10 g reared in the institute vivarium were taken for our study. The animals were kept under standard conditions with free access to food and water ad libitum. The ether used for anesthesia and euthanasia.

The source for the cell culture was brain of E14 rat embryos (14 dpc). Primary cell culture of rat fetal brain was obtained according to the standard protocol [27]. Fetal membranes were removed in the physiological saline and brain tissue suspended by repeated pipetting in DMEM medium (*Sigma*, Germany). Cells were centrifuged at 1500 rpm for 5 min and washed in the DMEM medium. Then fresh DMEM medium was added to the cell pellet and resuspended. The cell viability was determined using 0.2 % trypan blue staining (*Merck*, Germany) [27].

Cells in the amount of $1 \cdot 10^6$ were placed on adhesive coverslips (1 cm²), treated with polyethylenimine (*Sigma*, Germany), which were placed in a Petri dishes (n = 12) and cultured in the DMEM medium supplemented with 1 % fetal bovine serum (*Sigma*, USA), 400 mg % glucose and 0.2 units/mL insulin (complete medium volume was 2 ml).

Cells were cultured in the CO₂-incubator under standard conditions (37 °C, 95 % humidity and 5 % CO₂) and observed by means of an inverted microscope Eclipse TS 100 (*Nikon*, Japan) with microphotographic registration. Nutrient medium was changed every 3 days, total cultivation period was 37 days. On the 2nd and 37th days the cultures were fixed in 10 % formalin and the cytological and immunocytochemical analyses were performed.

Immunocytochemical staining on TGF- β 1 and nestin was performed according previously adapted technique. The primary antibodies were murine monoclonal antibodies for TGF- β 1 (*Sigma*, USA) and rabbit affine antibodies to nestin (*Sigma*, USA) at dilution 1:100. Secondary antibodies were goat anti-mouse/anti-rabbit IgG labeled by peroxidase (*Dako*, Denmark) at dilution 1:200. The immunocytochemical reactions were visualized using substrate solution with diaminobenzidine (*Dako*, Denmark), additionally stained with hematoxyline and embedded in the balsam. Additionally positive and negative controls were used. Rat fetal brain cells were used as negative control for which addition of primary antibodies was excluded. Cultured cells of rat glioma C6 were used as a positive control for TGF- β 1 (Bank of Cell Lines from Human and Animal Tissues, R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology National Academy of Sciences of Ukraine, Kyiv, Ukraine).

Microscopic investigation and photo registration of the cytological specimens of primary cultures were performed on the microscope AxioImager A2 (*Carl Zeiss*, Germany) with a wide-pass filter and photo camera AxioCam MRc5 (*Carl Zeiss*, Germany). Measurement of cells was performed in 10 representative visual fields with a standard measuring scale of stage micrometer. Analysis of digital images was performed by Zen Lite 2012 software (Germany). Total cell number, the number of immunopositive and negative cells, and morphometric indices were determined. The morphometric analysis was performed with digital image processing of cultures in 10 randomly fields of view (0.04 mm²) for each sample at the same magnification (x800). We determined: the number of cells, cross-section area of cell nuclei and cross-section area of the cell cytoplasm. The nuclear-cytoplasmic ratio (N/C ratio) was calculated by dividing the nuclear area by the cytoplasmic area.

The numbers of cells positive to TGF- β 1 and nestin were calculated in 10 randomly fields of view for each sample at the same magnification (x800) and calculated as a part of the total number of cells in percent.

Statistical analysis of the data was performed using Statistica 8.0 software (*StatSoft Inc.*, USA). We used the parametric (Student's t-test, two sample t-test with unequal variance) and non-parametric (Mann-Whitney U-test for comparison of independent groups) methods. As well as the Spearman's rank correlation were used. Normality of data distribution was defined according to the Shapiro-Wilk test. The differences were statistically significant at $p < 0.05$ and statistically highly significant at $p < 0.01$.

RESULTS AND DISCUSSION

To analyze subpopulations of the neurogenic cells culture of the rat fetal brain (E14), we conducted an immunocytochemical assessment of nestin expression. On the 2nd day of cultivation the nestin-positive cells made 49.16 ± 10.60 % of total cell number in the culture (**Fig. 1-A**).

Since nestin is a protein of intermediate filaments expressed in the NSCs and assumed to be a neuronal marker [28, 29], it seems reasonable to assert that neurogenic cell culture of the rat fetal brain (E14) contains on the average ~50 % of NSCs/NPCs. Mean values of the section area of the nestin-positive cells make 20.26 ± 0.83 μ m²; average values of nucleus section area 10.69 ± 0.33 μ m²; and nuclear cytoplasmic ratios 1.24 ± 0.06 .

At the same time, on the 2nd day of cultivation under standard conditions the number of TGF- β 1-positive cells in the primary cell cultures of the normal fetal brain of the rats (E14) made 22.04 ± 2.33 % (**Fig. 1-C**).

Morphometric indices of the TGF- β 1-positive cells in cell cultures of the fetal rat brain (E14) statistically differed on the 2nd day of culture from the indices of the TGF- β 1-negative cells. Average values of cross-section area of TGF- β 1-negative and positive cells in the culture of fetal brain of rats made respectively 31.38 ± 2.56 μ m² and 40.42 ± 2.89 μ m² ($p = 0.04$, Mann-Whitney U-test; **Fig. 2-A**); the average values of cross-section area of the nucleus made respectively 9.34 ± 0.47 μ m² and 14.58 ± 0.85 μ m² ($p = 0.003$, Mann-Whitney U-test; **Fig. 2-B**).

The nuclear-cytoplasmic ratio in the TGF- β 1-positive cells (0.62 ± 0.04) was higher than the index of negative cells 0.52 ± 0.05 ($p = 0.03$, Mann-Whitney U-test; **Fig. 2-C**). Thus, the sizes of the TGF- β 1-positive cells and of their nuclei in the cell cultures of rat fetal brain (E14) were greater than the sizes of the TGF- β 1-negative cells.

The correlation analysis confirmed the absence of significant association between quantitative indices of nestin- and TGF- β 1-positive and negative cells ($p > 0.05$, Spearman's rank coefficient).

During cultivation the total number of cells increased on the average by ~1.4 times. However the difference was not statistically significant ($p = 0.6$, Mann-Whitney U-test). At the same time the relative number of nestin-positive cells was somewhat decreased (37.78 ± 12.17 %, $p = 0.24$, Mann-Whitney U-test). By the 37th day of cultivation the relative number of TGF- β 1-positive cells also decreased, although these changes were not statistically significant (15.27 ± 9.80 %, $p = 0.7$, Mann-Whitney U-test).

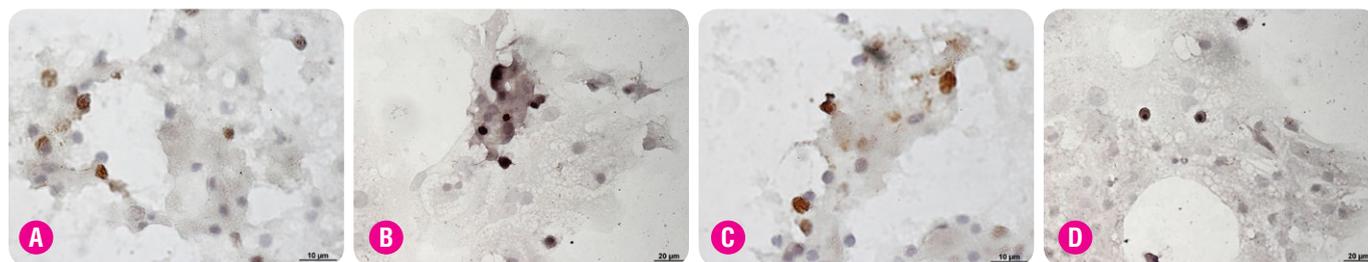
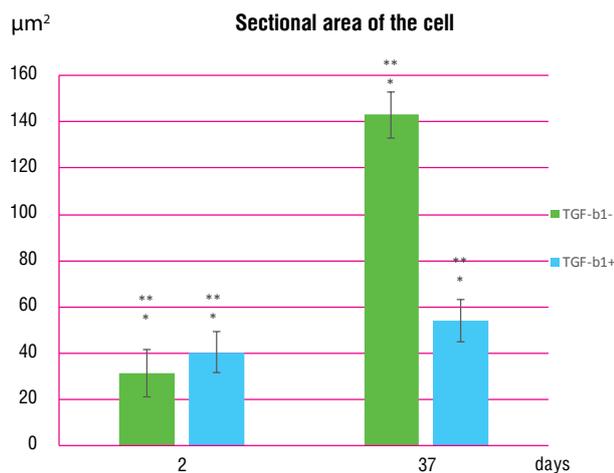
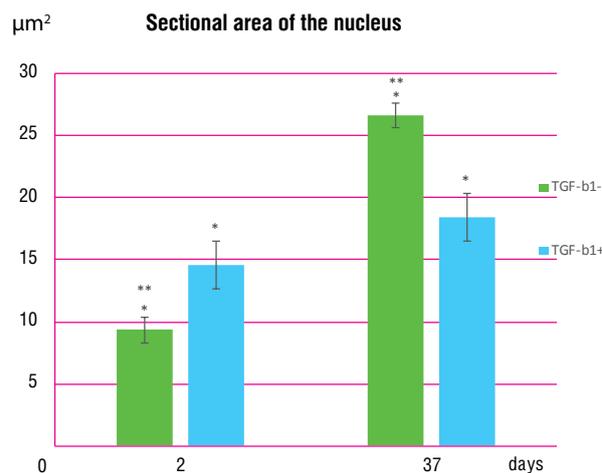


Fig. 1. Microphotographs of rat fetal brain cell cultures on 2nd (A, C) and 37th day (B, D) of cultivation. Immunocytochemical staining (brown) for nestin (A, B) and TGF-β1 (C, D) plus hematoxylin staining.

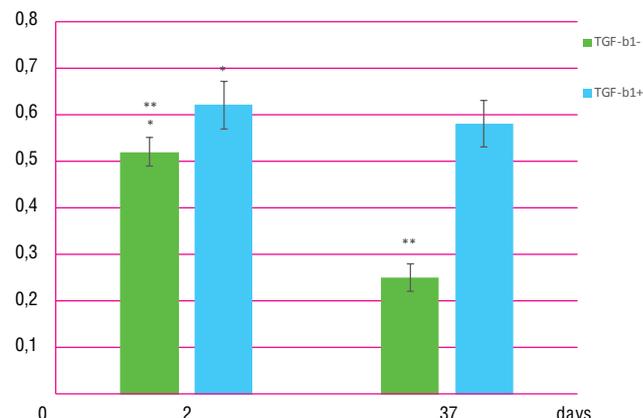


A



B

Nuclear-cytoplasmic ratio



C

Fig. 2. Morphometric characteristics of TGF-β1-positive and TGF-β1-negative rat fetal brain neurogenic cells (E14), n = 100: a – cross-sectional area of cells; b – cross-section area of nuclei; c – nuclear-cytoplasmic ratio (N/C ratio).

Notes:

* – $p < 0.05$ compared with TGF-β1-positive and negative cells;

** – $p < 0.05$ compared with 2nd and 37th days.

In view of the fact that nestin during NSCs differentiation is replaced by type-specific cells filaments – neurofilaments and glial fibrillary acidic protein (GFAP) [28, 29], we assumed that certain part of cells in fetal brain culture during 5 weeks starts differentiating. The evidence of changes of the morphometric indices of the cells on 37th day of speak in the favor of this assumption.

Mean values of section area of TGF-β1-negative and positive cells in fetal brain culture increased respectively $143.14 \pm 14.59 \mu\text{m}^2$ ($p = 0.000006$) and $54.03 \pm 8.57 \mu\text{m}^2$ ($p = 0.04$) in the comparison with the 2nd day indices and differed between themselves significantly ($p = 0.00003$, Mann-Whitney U-test).

Mean values nucleus section area of the TGF-β1-negative and positive cells in the rat fetal brain culture were respectively $26.62 \pm 1.05 \mu\text{m}^2$ and $18.43 \pm 2.83 \mu\text{m}^2$ ($p = 0.03$ in the comparison with those between positive and negative cells; $p = 0.000006$, $p = 0.14$ in the comparison with the 2nd day, Mann-Whitney U-test). The nuclear-cytoplasmic ratio in the TGF-β1-positive cells of fetal brain culture (0.58 ± 0.11) was higher than that in the negative cells (0.25 ± 0.03), $p = 0.004$, Mann-Whitney U-test. Thus, after one-month cultivation the sizes of cells in rat fetal brain cultures (E14) increased compared with 2nd day indices of cultivation. The sizes of TGF-β1-positive cells as well as their nuclei in the cultures were smaller compared with the sizes of the TGF-β1-negative cells.

Thus our investigation showed that the cultures of neurogenic cells of the rat fetal brain (E14) contained on the average 22 % of the cells expressing TGF-β; after one-month cultivation the TGF-β expression was found in nearly 15 % of culture cells. That is, a certain part of fetal brain cells kept TGF-β expression during 5-week period of cultivation under standard conditions.

As has been mentioned above, several authors presented the data regarding TGF-β1 expression by the fetal brain cells [18, 24–26]. As for biological role of TGF-β, it is assumed that under physiological conditions it plays an important role at all stages of ontogenesis: during embryo- and morphogenesis, and in the maintenance of homeostasis of the already formed tissues [13, 18]. It is assumed that TGF-β1-3 isophorms in embryogenesis of mammalian acts by both paracrine and autocrine mechanisms; regulate differentiation (stimulating or inhibiting depending on the type of cells), stimulate extracellular matrix production, act as chemoattractant for certain cells as well as induce

mesoderm formation at early life stages [18]. In the mature brain the TGF- β 1 is expressed at high level, regulating neurogenesis [30]. TGF- β 1 is a powerful inhibitor of NPCs proliferation *in vitro* [31] and promotes age-related decline of NPCs proliferation in the subventricular zone of the brain *in vivo* [32].

However attention of the investigators to TGF- β 1 is linked with its controversial role at the oncological processes. It is known that TGF- β 1-signaling is involved in the regulation of proliferation, differentiation and survival or apoptosis [14], modulates escape from immune control [16, 19-21], and presents a powerful inhibitor of the proliferation of immune cells [16, 22]. Expression of TGF- β isoforms is elevated in the gliomas with high malignancy degree [11-16], promoting avoidance by tumor of immune recognition with means of various mechanisms, including such as CD8⁺ CTL and natural killers inhibition [23], and stimulation of the generation of T-regulatory cells.

As has been mentioned, the elements of TGF- β -signaling are considered to be potential targets for anti-tumor therapy [11-16, 33]. In parallel, new approaches with the use of NSCs/NPCs are being developed for treatment of gliomas of brain [1-8]. In our previous investigations we demonstrated certain aspects of anti-tumor and immunomodulating properties of the fetal brain neurogenic cells, specifically the humoral factors produced by them (rat NPCs supernatant) [34-36]. Since in the present work we confirmed the presence of the TGF- β 1 expression in part of the neurogenic cells of fetal brain and in view of possible paracrine and autocrine mechanisms of its action [18], we do not exclude that this particular fraction of TGF- β 1-positive cells is the carrier of anti-tumor and immunomodulating properties of rat NPCs and can be used in the elaboration of cell therapeutic methods of cell therapy for brain gliomas. It is our hope that further exploration of the role of TGF- β 1 in anti-tumor and immunomodulating effects of rat NPCs is perspective.

CONCLUSIONS

We have found the presence of TGF- β 1 expression in part of the neurogenic cells of rat fetal brain (E14) *in vitro*, which was kept during nearly 5 weeks of culture.

We have established the significant quantitative differences of the morphometric indices in the TGF- β 1-positive and negative cells of rat fetal brain (E14) in the dynamics *in vitro*.

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