Retinoic acid (RA) plays important roles in development, growth, and differentiation by regulating the expression of its target genes. The pro-apoptotic Bax gene may form channels through oligomerization in the mitochondrial membrane and facilitate the cytosolic release of cytochrome c. The anti-apoptotic Bcl-2 gene can inhibit this process. Up-regulated gene 4/Upregulator of cell proliferation (URG4/URGCP) is a novel gene located on 7p13. URG4/URGCP also stimulates cyclin D1 (CCND1) mRNA expression, and RNAi-mediated URG4/URGCP silencing diminishes CCND1 mRNA expression in HepG2 cells. In this study, the effects of RA treatment on URG4/URGCP, CCND1, Bcl-2 and Bax gene expression changes in undifferentiated and differentiated SH-SY5Y neuroblastoma cells was analyzed. SH-SY5Y cells were cultured in the appropriate conditions. To induce differentiation, the cells were treated with 10 micromolar RA in the dark for 3-10 days. SH-SY5Y cells possess small processes in an undifferentiated state, and after treatment with RA, the cells developed long neurites, resembling a neuronal phenotype. Total RNA was isolated with Tri-Reagent. According to the results, Bcl-2 and CCND1 gene expression levels were increased, while URG4/URGCP and Bax gene expression was decreased in RA treated cells compared to the control cells. Our preliminary results suggest that RA may induce cell proliferation and escape apoptosis using a novel pathway by the URG4/URGCP gene. Further investigations are needed to clarify more direct transcriptional targets of RA signaling and the interaction of RA pathways with other pro-regenerative signals.

Key words: retinoic acid, URG4/URGCP, human neuroblastoma cells, SH-SY5Y.

Expression of URG4/URGCP, Cyclin D1, Bcl-2, and Bax genes in retinoic acid treated SH-SY5Y human neuroblastoma cells

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Introduction

Neuroblastoma (NB) is the most frequent extra-cranial solid malignant tumor found in childhood cancers [1]. Approximately half of all children with NB are classified as high-risk patients, despite intensive therapeutic cures. In view of these cases, any effort to improve diagnosis and therapy is of great clinical interest.

Retinoids are involved in the proliferation, differentiation, and apoptosis of various cell types. Active retinoids occur in three forms: alcohol (retinol), aldehyde (retinal or retinaldehyde), and acid (retinoic acid – RA). Retinoic acid plays important roles in development, growth, and differentiation by regulating the expression of its target genes. Retinoic acid appears to directly regulate more than 500 proteins [2–4]. The role of retinoids as agents inducing differentiation has been under investigation, and their use in the diet remains a promising therapy for the prevention of several types of cancer [5–7]. Neuronal differentiation can be induced in vitro in NB cells by exposure to RA, which is the most commonly used compound for differentiation. Sidell determined that RA can induce growth inhibition and morphologic differentiation of human NB cells in vitro in 1982 [8]. The differentiating inducer RA can drive the cells in different directions during their maturation. Additionally, it is discussed that the dramatically different response to RA is dependent on the tumor stage of the patient. Thus, aggressive and spreading tumor cells could be transformed in vitro to mature and non-proliferating cells [9, 10].

Apoptosis is a complex biological mechanism that organisms use to eliminate unwanted cells. Specialized cellular receptors and signals initiate the apoptotic machinery and a complex set of reactions leads to characteristic changes. Inhibition of apoptosis in the tumor cells would lead to cellular immortality. The Bcl-2 family of proteins has expanded significantly and includes both pro- as well as anti-apoptotic molecules. Bcl-2 family proteins are important regulators of apoptosis. They consist of a wide variety of anti-apoptotic proteins such as Bcl-2, but also include pro-apoptotic proteins such as Bax. Bcl-2 has been shown to promote cell survival. Bax, a pro-apoptotic protein member of the Bcl-2 family, participates in the induction of apoptosis in response to many apoptotic signals. Over-expression of Bax has been shown to induce apoptosis in a variety of different cellular contents [11, 12]. Bcl-2 is a pro-survival protein that resides on the mitochondrial membrane and prevents apop-
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RNA extraction and semi-quantitative reverse transcription PCR

Total RNA was isolated with Tri-Reagent (Sigma, St. Louis, MO, USA) according to the manufacturer’s instructions with minor modifications and quantitated with an Nanodrop™ spectrophotometer (Thermo Scientific). RT reaction was performed using the First-Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer’s protocol. Appropriate cycles were chosen to ensure the termination of PCR amplification before reaching a stable stage in each reaction. Gene expression was presented as the yield of PCR products from target sequences relative to the yield of PCR products from the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. PCR primers and reaction parameters are listed in Table 1. Semi-quantitative PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining and photographed under UV light. Gene expression was presented as the yield of PCR products from target sequences relative to the yield of PCR products from the GAPDH gene. In each instance, the amount of reverse transcription (RT)-PCR product for the gene of interest was normalized to the amount of GAPDH in the same sample. The experiments were repeated twice in duplicate in each group.

Results

The SH-SY5Y cells were initially treated with RA for 3–10 days. SH-SY5Y cells possess small processes in an undifferentiated state, and after treatment with RA, the cells developed long neurites, resembling a neuronal phenotype [20].

Material and methods

SH-SY5Y cell culture and differentiation

Human SH-SY5Y neuroblastoma cell line was grown in DMEM-Ham’s F12 medium supplemented with 2 mM L-glutamine, penicillin (20 units/ml), streptomycin (20 μg/ml), and 10% (vol/vol) heat-inactivated fetal calf serum at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO2. During culture, media were changed every 2 days and cells were replated before confluency. All experiments were conducted with exponentially growing cells. To induce differentiation, the cells were treated with 10 micromolar RA [19] in the dark for 3–10 days. SHSY5Y cells possess small processes in an undifferentiated state, and after treatment with RA, the cells developed long neurites, resembling a neuronal phenotype [20].
ium bromide. The A260/A280 ratio was between 1.9 and 2.0.

The effect of RA on URG4/URGCP, Cyclin D1, Bcl-2, and Bax gene expression is shown in Fig. 2. Changes in mRNA levels, detected using semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR), were calculated as the proportion of the target gene amplification products to the amplification products of the housekeeping gene GAPDH. According to our results, Bcl-2 and CCND1 gene expression was increased in RA treated groups, while URG4/URGCP and Bax gene expression was decreased in SHSY5Y cells after RA treatment compared with the control cells (Fig. 2).

Discussion

Neuroblastoma (NB), which arises from cells of the neural crest, is the most common extra-cranial tumor of childhood. The behavior of neuroblastoma has puzzled investigators and is unique among malignant neoplasms. Defining the mechanisms responsible for its behavior may lead to the eventual successful treatment of this tumor. It is known that there has been increasing interest in recent years in the application of differentiation inducers in the therapy of NB. The genes and pathways that mediate the biological effects of RA have not been fully elucidated.

Retinoic acid is one of the antitumor agents that has been used successfully to treat certain human tumors including neuroblastomas [21]. Indeed, NB patients treated with RA have an increased survival rate without severe side effects [22]. Accumulating evidence suggests that RA plays an important role in the regulation of NB apoptosis as well as differentiation. However, certain neuroblastomas display an RA-resistant phenotype [23, 24]. To further improve the therapeutic effects of RA on neuroblastomas, it is necessary to clarify the detailed molecular mechanisms underlying the RA-mediated neuroblastoma differentiation and/or apoptosis.

Cell apoptosis is the process of programmed cell death that involves a series of biochemical events leading to a characteristic cell morphological change and death. Under physiological and pathological conditions, in the absence of such survival factors, cells enter a gene regulated program of self-elimination which depends on RNA and protein synthesis and on the expression of a specific set of genes. Apoptosis uses several genes related to the cell cycle and a very exciting point is the dependence of apoptosis on cell cycle phases. In our hands there is multiple cell cycle access to apoptosis. Various human NB cell lines express oncoproteins of the Bcl-2 family. These protein components of the apoptotic regulatory machinery control neuronal survival. Some of these components, such as Bcl-2 and Bak, promote it [12, 25]. In mitochondrial pathways of apoptosis, Bcl-2 family proteins are critical determinants of mitochondrial membrane potential, which controls the cytoplasmic release of cytochrome c from mitochondria, thereby regulating apoptotic cell death. They are divided into two subfamilies based on their biological roles. The anti-apoptotic subfamily includes Bcl-2 and Bcl-xL, while the pro-apoptotic subfamily includes Bax, Bim, and Bmf. The balance between these two groups determines the fate of cells. Anti-apoptotic Bcl-2 is one of the most important members that inhibit the mitochondria-dependent apoptotic pathway triggered by diverse cytotoxic agents through blocking mitochondrial permeability transition.

![Effect of RA on morphology of SHSY5Y cells.](image_url)

Fig. 1. Effect of RA on morphology of SHSY5Y cells. The cells were induced to differentiate for 10 days by adding 10 micromolar (final concentration) RA in medium containing 10% heat-inactivated fetal bovine serum. After every 24 h, medium was replaced with fresh medium containing 10 micromolar RA. A) Untreated cells (Olympus CKX41-X10); B) RA-treated cells (Olympus CKX41-X10).
sion of pro- or anti-apoptotic proteins of the Bcl-2 family modifies the sensitivity to induced apoptosis. The overexpression of anti-apoptotic Bcl-2 in neuronal cells was shown to prevent programmed cell death both in vitro and in vivo [26].

URG4/URGCP, a novel gene up-regulated in human hepatocellular carcinoma and gastric cancer, has been identified recently. URG4/URGCP is located on chromosome 7 (7p13). Previous data have suggested that URG4/URGCP might be an oncogene operating in hepatocarcinogenesis [14] and gastric carcinogenesis [16]. URG4/URGCP expression was found to be up-regulated in gastric cancer tissues compared with matched adjacent non-neoplastic tissues. In vitro observation showed that URG4/URGCP is up-regulated in gastric cancer cell lines compared with normal gastric epithelial cell lines, suggesting that URG4/URGCP might play an oncogenic role in the development of gastric cancer [16]. CCND1 is a periodic regulatory protein that is believed to govern cell cycle transit from G1-phase into S-phase, and has been found to be abnormally expressed in many human cancers. Overexpression of CCND1 leads to abnormal cellular proliferation, which underlies the process of tumorigenesis. Thus, CCND1 can function as a cooperative oncogene in cell transformation. However, until now, there has been no investigation on URG4/URGCP expression in NB and also RA.

In our experiments, we investigated the influence of RA on a novel URG4/URGCP gene, and CCND1, Bcl-2, Bax gene expression changes in undifferentiated and differentiated SH-SY5Y neuroblastoma cells were analyzed. The results showed that Bcl-2 and CCND1 gene expression was increased in RA treated groups from the 3rd to 10th days; and also URG4/URGCP and Bax gene expression was decreased in SH-SY5Y cells after RA treatment compared with the control cells from the 3rd to 10th days. This study demonstrates the relationship between states of differentiation, and relative levels of URG4/URGCP, CCND1, Bcl-2, and Bax gene expression in neuroblastoma cells. To our knowledge, this is the first reported study of time-dependent changes in the gene expression of a human neuroblastoma cell line treated with RA. We suggest that RA may induce cell proliferation and escape apoptosis using a novel pathway by the URG4/URGCP gene. Further investigations are needed to clarify more direct transcriptional targets of RA signaling and the interaction of RA pathways with other pro-regenerative signals.

The authors declare no conflict of interest.

References