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Retinoic Acid Stimulate Differentiation of Hippocampal Stem Cells into Opsin Expressing Cells *in vitro*

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Abstract: The results of several studies have demonstrated that cell differentiation influenced by derivatives of retinoic acid. To determine whether retinoic acid mediate the differentiation of neural stem cells we treated dissociated hippocampal stem cells with different concentrations of all trans or 9-cis retinoic acid and analyzed the effects on cell fate by specific monoclonal antibody for photoreceptors. Addition of exogenous retinoic acid caused a dose dependent specific in the elevation of the cell number that developed as photoreceptors in culture. Also results of immunohistochemical studies using monoclonal antibody demonstrated that the primary effect of retinoic acid was to influence progenitor cells the developed as mature and immature photoreceptors. These results suggest that retinoic acid may play an important effect in the normal development of photoreceptor cells *in vitro*.

Key words: Retinoids, hippocampus, photoreceptors, rat development

INTRODUCTION

Vitamin A derivatives such as all-Tran's retinoic acid (ATRA) are known to profoundly influence cell differentiation, survival and lineage decision making observations of ATRA-induced teratogenesis (Bohmer *et al*., 2001; Akagawa, 1990). Subsequent reports on the spatiotemporal patterns of retinoic receptor expression suggest that the developing nervous system is a major target for ATRA actions (Banker and Cowan, 1997; Blondel *et al*., 2000). In most mammals, including rats the generation of the different classes of retinal cells can be roughly divided into early and late phases. In the early phase, ganglion cells, horizontal cells, cone cells, amacrine cells are born, while most rod photoreceptors Muller and bipolar cells are born in late phase of histogenesis (Watanabe and Raff, 1998).

Although the specific factors that promote differentiation of cells as rod photoreceptor not known, several classes of molecules have been shown to influence cell determination in other developing systems and might also play a role in the developing retina (Bence *et al*., 2001; Chapple *et al*., 2001). For example small peptide growth factors have been shown to play a role in the developing nervous system (Anchant *et al*., 1991; Dupin and Le Douarin, 1995). Among the other classes of molecules that have been shown to influence cell fate in the developing nervous system is the vitamin a derivative retinoic acid (Tezel *et al*., 1999). In particular, ATRA has been shown to alter cell fate decisions in the developing limb bud, hind brain and inner ear (Kelley *et al*., 1994). More recently, 9-cis isomer of retinoic acid has been shown to biological effects that are apparently mediated through the RXR family of nuclear receptors. In addition several studies have demonstrated that both all-trans and 9-cis retinoic acid and at least one of the nuclear retinoic acid receptors (RARα) are present in the developing retina (Kelley *et al*., 1994).

Retinoids including all Trans' retinoic acid can influence the survival of cells dissociated from E8 chick retina, a stage when neurogenesis is largely complete (Chalmers-Redman *et al*., 1997; Yan and Wang, 2000).

Therefore, we determined the effects of retinoic acid on the differentiation of Hippocampal stem cells *in vitro*.

MATERIALS AND METHODS

Animals: The animal experiments were conducted in Pharmacology and Anatomy Department and Western blotting works were conducted in Cellular and Molecular

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Research Center of Iran Medical University from 2004-2007. All animals were obtained from breeding colony of Iran University of medical sciences, Tehran, Iran. Male Wistar rats (215±10 g) were allowed to mate with female virgin wistar rats (215±10 g) during a 24 h period. Female rats were checked for the presence of a vaginal plug twice at midnight and at 7 am the next day. Once the vaginal plug was observed the animal considered as pregnant. The pregnant rats were housed individually in cages with a 12 h light/dark cycle at 22-24°C temperature, with food and water *ad libitum*. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the care and use of laboratory animals and all experiments conformed to Iran University of Medical Sciences guidelines on the ethical use of laboratory animals. Also in each experiment care was taken to minimize the animals suffering and to use the minimum number of animals.

Cell culture: Hippocampi were removed from 6 (n = 6) rat embryos in embryonic day 18 (E18) and mechanically dissociated with a fine-tipped pasture pipette in Hanks Buffer Slain Solution (HBSS). At the end of the separation, the cells were counted and assessed for viability using Trypan Blue dye exclusion. After that cells plated into 6 well plates (falcon) at a density 2×10^5 cells per well. The wells were coated with inactive astrocytes (method of mc carthy and develis) (McCarthy and DeVellis, 1980; Kang *et al*., 2001). The culture medium contained DMEM/F12 (1-1), 25 μg mL^{-1} insulin, 100 μg mL^{-1} transferin, 60 μM putresine, 30 nM selenium, 20 nM progesterone, 100 unit mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 10% Fetal Bovine Serum (FBS) (Gibco-BRL). One half of the media in each well was changed every 3 days. Stock solutions of 1 mM all-Trans and 9-cis retinoic acid were made in dimethyl sulfoxide (DMSO) and stored in-70°C in the dark. Individual aliquots were thawed and diluted in culture medium directly prior to use. Each well exposed to different concentrations of ATRA or 9 Cis-RA for 8 days. All control groups contained same culture medium without any concentration of ATRA or 9 Cis-RA and the all wells coated with inactive astrocytes.

Immunocytochemistery: To identify specific types of retinal cells in each of the wells, plates were processed for Immunocytochemistery using a previously published protocole (Keljavin and Reh, 1991). At first cells were fixed at 6 day *in vitro* (DIV) with 4% Para formaldehyde for 20 min. Following three 5 min rinse in Phosphate Buffer Salin (PBS), primary antibodies were diluted in PBS containing 10% normal goat serum and 0.3% Triton X-100 (sigma) and incubated at 37°C for 2 h. Monoclonal anti Opsin (sigma) was used at 1:1000 following further rinses in PBS and incubated in secondary antibody conjugated with alkaline phosphatase 1:500 for 1 h. After removal of the secondary antibody substrate of BCIP/NBT were applied at room temperature for 30 min. Samples were then washed and examined using an inverted microscope (Olympus).

Western immunoblotting: Antibody used for western blot analysis was antiopsin Ret P1 (sigma). Differentiated cells in plates were harvested with 0.5% trypsin EDTA and the cell pllet was suspended in ice-cold lysis buffer (2 mM HEPES, 2 mM EDTA, 50 μm phenyl methyl sulfonyl fluoride, 1 μg mL^{-1} aprotinin, leopeptin) for 15 min and subsequently centrifuged at 1200 RPM for 30 min at 4°C after which the middle layer was collected, after an aliquot was taken for the protein determination by the lowry method (Lowry *et al*., 1954), samples were stored at -70°C until ready for electrophoretic analysis, electrophoresis was performed according to Laemmli method (Leammli, 1970). Gels were equilibrated in transfer buffer and then electro blotted on nitrocellulose membrane (0/22 μm) and then monoclonal antiopsin Ret P1 antibody was done for the membrane. Secondary antibody conjugated with alkaline phosphatase. Western blot analysis (Fig. 5) indicated that there were different forms of opsin protein (~40 kDa) (arrow head), (>41 kDa), (~60 kDa) and (~30 kDa).

Statistical analysis: The data obtained from counting of stained cells in the flask and analyzed by using SPSS version 12. Then, variables have been analyzed by one-way Analysis of Variance (ANOVA) followed by Tukey post hoc test. All data were expressed as the Mean±SEM and the differences were considered to be significant when $p<0.05$.

RESULTS AND DISCUSSION

Results indicate that addition of all-Tran's retinoic acid (ATRA) to the culture medium had a striking effect on the development of opsin expressing cells. After 6 days *in vitro*, cultures that were maintained in medium that contained either 100 to 500 nM ATRA developed a significantly greater number of opsin expressing cells in comparison with controls. The morphology of these cells was reminiscent of developing photoreceptors *in vivo* including a thin axonal like process and wider process more similar to the inner segment and developing other segment (Fig. 1a, b).

Effect of all-trans retinoic acid: Figure 2 shows the results as function of different ATRA concentrations. The number of rhodopsin immunoreactive cells were

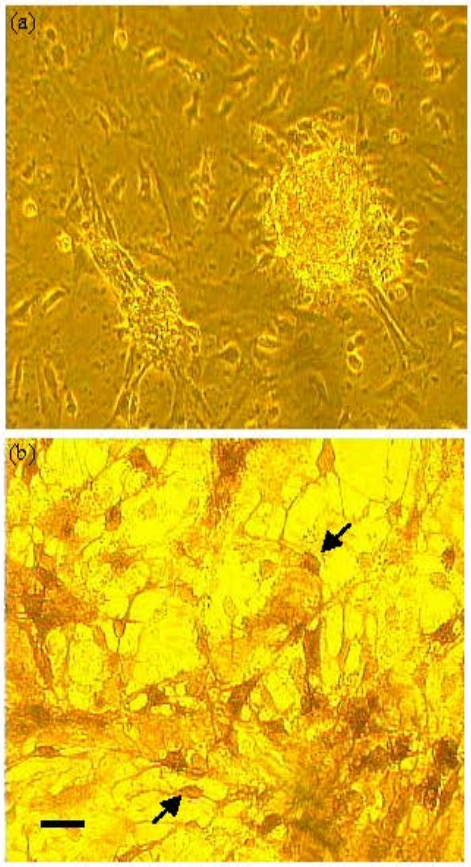

Fig. 1: Micrographs of immunoreactive opsin expressing cells in retinoic acid-treated culture (a, b). Note that retinoic acid cause An increase in the number of opsin expressing cells. Scale bar in B (same in A), 50 μm

significantly greater ($\alpha = 0.025$) in culture treated with ATRA at concentration of 100 nM. The number of opsin expressing cells was also elevated in culture related with 100 nM AT retinoic acid as compared with control groups. The greatest increase in the number of opsin expressing cells was in culture wells treated with 500 nM AT retinoic acid, while the number of opsin expressing cells in cultures maintained in medium with 1.0 μM AT retinoic acid was lower than 100 nM At retinoic acid (Fig. 2).

Effect of 9-cis retinoic acid: Recent studies have demonstrated that the preferred ligand for the retinoic X receptors (RXR) is the 9-cis, isomer of retinoic acid. Since a percentage of AT retinoic acid can be isomerized to 9-cis retinoic acid *in vivo*. Therefore, the E18 experiments were repeated using exogenous 9-cis rather than all-trans retinoic acid. Cultures of E18 Hippocampal cells that were exposed to 9-cis retinoic acid for 6 days responded similarly to E18 cultures that had been exposed to AT

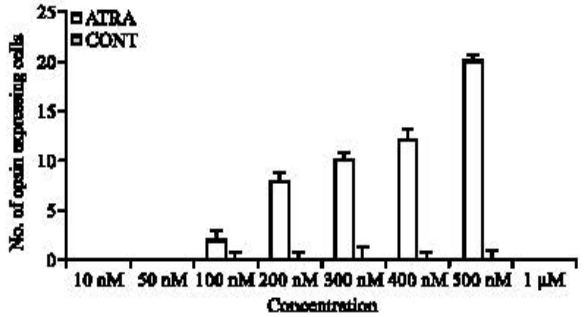

Fig. 2: Response of hippocampal cultures established on E18 to different concentration of all-trans retinoic acidafter 6 DIV. Retinoic acid cause a significant dose dependent increase in the number of cells that developed as opsin expressing cells. The total cell number did not change between cultures with different concentration of retinoic acid but the number of cells that differentiated as rod opsin expressing cells increased significantly with concentration (400-500nM) ($\alpha = 0.025$). Data are from the results of five separate experiments. Error bars are standard error of the mean (SEM)

retinoic acid (Fig. 3). There was a dose dependent increase in the number of cells. However, the magnitude of the increase in the number of opsin expressing cells was significantly higher in cultures treated with 9-cis retinoic acid. In cultures treated with 100 nM 9-cis retinoic acid, the total number of rod cells increased over control values. The time course of the effect of retinoic acid and 9-cis retinoic acid on the differentiation of opsin expressing cells was determined by fixing hippocampal stem cells after progressively longer periods of time in cultures. E18 hippocampal cells were dissociated and maintained in cultures at high density with 500 nM AT retinoic acid or 100 nM 9-cis retinoic acid.

Figure 4 shows in both AT retinoic acid and 9-cis retinoic Acid treated there was no rhodopsin immunoreactive rods after 2 days of cultures. However by 6 and 8 days *in vitro* there was already a significant increase over control wells in the number of opsin expressing cells in the retinoic acid treated wells (Fig. 3-5).

In the present study we provide novel evidences that ATRA and 9-cis RA has an important role to differentiation of neural stem cells into opsin expressing cells. Results indicate a time dependent activity in both forms of retinoic acid. We revealed that there are three kinds of photoreceptors (mature, dimmer and deglycosylated) after differentiation. Therefore, present finding showed that retinoic acid has an important role to produce opsin expressing cells from adult stem cells.

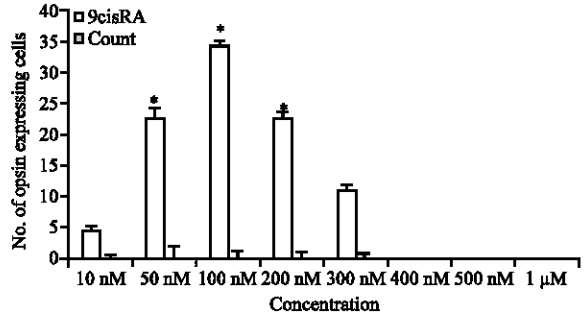

Fig. 3: Response of hippocampal cultures established on E18 to different concentration of 9-cis retinoic acid after 6 DIV. 9-cis retinoic acid cause a significant dose-dependent increase in the number of cells that developed as opsin expressing cells. Addition of 100 nM 9-cis retinoic acid results in a significant in the number of cells that differentiate as opsin expressing cells. however, the magnitude of the increase in the number of opsin expressing cells was more than the response to an equivalent concentration of all-trans retinoic acid. The total number of cells that developed as opsin expressing cells in response to 9-cis retinoic acid in compression with ATRA is very significant. Data are from the results of five separate experiments. Error bars are standard error of the mean (SEM)

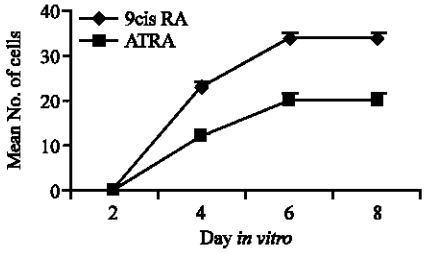

Fig. 4: Time course of all-trans retinoic acid and 9-cis retinoic acid effects on differentiation of opsin expressing cells. Addition of 500 nM ATRA and 100 nM 9cis RA results in a significant increase in the number of cells that differentiate as photoreceptors. In retinoic acid treated cultures, development of opsin expressing cells appears to be shifted ahead by approximately 48 h

In recent years, several studies have shown that cell-cell interactions are necessary for the appropriate differentiation of vertebrate cells *in vivo* (Azuma *et al*., 2000) and *in vitro* (Kiuchi *et al*., 2002; Sharma *et al*., 2001). ATRA are known to profoundly influence cell differentiation, survival and lineage

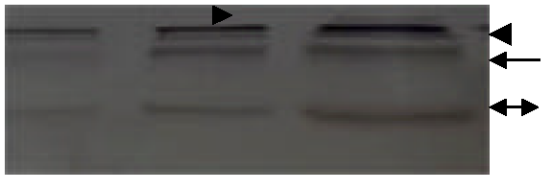

Fig. 5: The electrophoretic mobilities of different glycoforms of opsin are indicated mature (~41 kDa) (◀) and immature forms (>41 kDa) and dimmer (~60 kDa) (◀▶) and deglycosylated form (~30 kDa) (←)

decision making. In culture of CNS, ATRA promotes the production of rod photoreceptor cells at the expense of amacrine cell (Kelly *et al*., 1994). these experiments have demonstrated that the differentiation of hippocampal stem cells into rod photoreceptors is influenced by locally diffusible signals in the extra cellular environment. These signals are developmentally regulated during the period of retinal histogenesis. Lillien and Cepko (1992) and Wohl and Weiss (1998), however the specific factors that determined or promote the photoreceptors have not be identified . In the present study, we have found that all trans retinoic acid and 9-cis retinoic acid can influence the development of rod photoreceptor cells *in vitro*. The number of photoreceptors also elevated in culture treated with 9-Cis RA as compared with control group. Our results are agreement with data of Kelly *et al*. (1994), Watanabe and Raff (1988) who showed 9-Cis RA increased the number of photoreceptors their results indicated the best concentration for differentiation was 100 nM and for ATRA was 500 nM . In culture of retinal cells that isolated from E15 retinas differentiated as photoreceptor at a higher level when embryonic cells co-cultured with post natal cells, indicating that differentiation mediated by a soluble factors that extracted from mature cells (Watanabe and Raff, 1988). In the embryonic cultures, retinoic acid appears to act directly on the mitotically active progenitor cells to direct their fate to the rod photoreceptor phenotype (Zhao *et al*., 1997). We have found a retinoic acid dose-dependent increase in the number of opsin immunoreactive cells in E18 cultures after six days *in vitro*. The total number of cells that were induced to differentiate as photoreceptors as a result or retinoic acid treatment was much lower in ATRA than in 9-cis retinoic acid. These results suggest that retinoic acid is probably not the only factor necessary for photoreceptor differentiation. However, a recent hypothesis has suggested that as development proceeds the potential phenotype choices of individual progenitor cells my be influenced based on changing

sensitivities to different factors (Zhao *et al*., 1997). If this hypothesis is correct, then retinoic acid might only have an effect on those progenitor cells that have become restricted to differentiate as later retinal phenotypes such as rod photoreceptors. The wild-type of opsin protein, has different forms such as mature, immature, deglycosylated and dimmers. Form of glycosilate plays pivotal roles in protein folding, oligomerization, quality control, sorting and transport. Inhibition of glycosilation blocks the synthesis of precursor oligosaccharides required for N- linked glycosilation. The form of agressomes may have important consequence for photoreceptor viability (Saliba *et al*., 2002). In the electrophoresis we have found 3 kinds of opsin protein with different molecular weight this show the differentiated cells are very similar to normal rod photoreceptor cells.

In conclusion, both all trans retinoic acid and 9-cis could differentiate neural stem cells into rod photoreceptor like cells and differentiated cells are like as normal cells.

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