RESEARCH ARTICLE

A combined opiate agonist and antagonist treatment reduces prolactin secreting pituitary tumor growth

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Abstract Prolactin secreting pituitary adenomas (prolactinomas) is the most common pituitary tumors in humans. Animal studies have identified aggressive prolactinoma development in fetal alcohol exposed rats. We have recently identified a combination treatment of a μ opioid receptor antagonist naltrexone and a δ opioid receptor agonist D-Ala2-,N-Me-Phe4,Gly-ol Enkephalin (DPDPE) increases innate immune function. In this study, we tested whether naltrexone and DPDPE combination therapy is useful to control pituitary tumor growth. Fetal alcohol exposed and control Fischer 344 female rats at 60 days of age were ovariectomized and received an estrogen implant to induce prolactinomas. Six weeks after the estrogen implant, these animals received treatments of naltrexone and DPDPE or saline. The growth of the pituitary tumor prior to and after opioidergic agent treatments was visualized using magnetic resonance imaging (MRI). At the end of the treatment, pituitary weights, plasma prolactin and splenic levels of cytotoxic factors were determined. Both imaging data and weight data indicated that the volume and the weight of the pituitary were increased more after estrogen treatment in animals exposed to fetal alcohol than control. Naltrexone and DPDPE treatment reduced the weight and volume of the pituitary gland and plasma levels of prolactin in both fetal alcohol exposed and control-fed animals. The treatment of opioidergic agents also increased the levels of cytotoxic factors in the spleen. These data provide a novel possibility in treating pituitary tumors using a combination therapy of naltrexone and DPDPE.

Keywords Pituitary prolactin secreting tumors · Estradiol · Fetal alcohol · Opioidergic drugs · NK cells

Introduction

Endogenous opioid peptides, such as endorphin, which is involved in body stress controlling mechanisms, have been shown recently to enhance immune function and prevent cancer growth and progression in various animal models of cancers (Boyadjieva et al. 2009; Sarkar et al. 2008, 2011; Zhang and Sarkar 2012; Sarkar and Zhang 2013; Murugan et al. 2014; Zhang et al. 2015). Therefore, targeting the endogenous opioid peptides receptors in the regulation of cancer chemoprevention may offer new promise for the design of therapeutic strategies. Opioid receptors have been shown to be present in various immune cells including natural killer (NK) cells (Boyadjieva et al. 2004). Acute treatments of opioid receptor agonists have been shown to stimulate NK cell cytolytic activity, and an opioid receptor antagonist block opioid peptide-stimulated NK cell function in the spleen (Boyadjieva et al. 2004; Boyadjieva and Sarkar 2010). Previously, we have shown that one of the very effective way to activate NK cell cytolytic function is to combine a μ opioid receptor antagonist naltrexone and δ opioid receptor agonist D-Ala2-,N-Me-Phe4,Gly-ol Enkephalin (DPDPE) because of opioid receptor dimerization in these immune cells (Boyadjieva et al. 2004; Sarkar et al. 2012). Combined treatments of naltrexone and DPDPE have shown to reduce N-methyl-N-nitrosourea (NMU)-induced mammary tumor growth in rats. Whether or not these treatments are effective in reducing the tumor growth in non-carcinogen induced tumors is not known (Sarkar et al. 2012).

In order to test the effectiveness of a combined opiate agonist and antagonist treatment in tumor suppression, we employed a rat model of pituitary prolactinomas. Prolactinomas

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are the most frequent pituitary tumors in adults accounting for 60% of all functioning pituitary tumors (Ciccarelli et al. 2005). Even though prolactinomas are usually benign and in most cases respond well to treatment with dopaminergic agents, 15% of these tumors are resistant to classical therapy, become invasive and aggressive, and require extirpation (Wong et al. 2015a, 2015b). Hence, there is a need to develop alternative therapies for these aggressive tumors. The estrogen-treated rat is a well-known model for prolactinoma development with increased pituitary weight, hyperprolactinemia and lactotroph hyperplasia (Heaney et al. 1999, 2002). Estrogen-treated fetal alcohol exposed rat provides an animal model of aggressive prolactinomas (Gangisetty et al. 2015). Studies in animal models of prolactinomas have been very helpful to identify molecular pathways altered in these tumors and to test potential future therapies (Heaney et al. 1999; Hentges and Sarkar 2001; De et al. 2002; Cristina et al. 2006; Sarkar 2006, 2010; Gangisetty et al. 2015). In this study, employing estrogen-treated fetal alcohol exposed female rats we investigated the effectiveness of a combined naltrexone and DPDPE treatment in suppressing the growth of prolactinomas in estrogen-treated fetal alcohol exposed rats.

Materials and methods

Animals and treatments Pregnant Fischer 344 rats were fed between gestational days 7 and 21 with a liquid diet containing 6.7% alcohol (AF), pair-fed with isocaloric liquid diet (PF), or fed ad libitum with rat chow (AD). Feeding this alcohol diet in pregnant rats has been shown to elevate about 100 mg/dl levels of blood alcohol (Miller 1992). At 60 days of age, female offspring rats were ovariectomized and implanted subcutaneously (sc) an estradiol-17 β capsule under isoflurane gas anesthesia. 6 weeks after the estrogen treatment, animal received s.c. implant of a placebo pellet (Control: 50 mg/pellet, 21 day release; Innovative Research of America, Sarasota, FL) or a naltrexone pellet (NALT: 50 mg/pellet, 21 day release; Innovative Research of America, Sarasota, FL). After a week of naltrexone or placebo implants, these animals were then received daily ip injection of DPDPE (100 μ g/kg weight of rat; Sigma-Aldrich; St. Louis, MO) or saline for 2 weeks. The growth of the pituitary tumor was visualized using magnetic resonance imaging (MRI) prior to and 21 days after the implants. MRI images were obtained by using M2 MRI Scanning System, 60 mm Radio Frequency Coil, T2 Weighted GRE-SP (Gradient Echo Spoiled) sequence, and NRG-console software. During scanning, the animals were anesthetized with 2,5% isoflurane mixed with oxygen (1 l/min). Vivo Quant – software was used for analyzing images. At the end of the treatment, pituitary weights and immune functions were determined.

Western blot After agonist and antagonist treatment, splenocyte suspensions were lysed in lysis buffer containing 150 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM MgCl₂, 1% Triton-X, 10% glycerol and protease inhibitor cocktail (Sigma, St. Louis, MO) for 1 h on ice. The cellular debris was cleared by centrifugation (10,000 g for 10 min). For cytolytic protein detection, equal amounts of protein from each sample were resolved on SDS-PAGE and transferred to immobilon-P-PVDF membrane (Millipore, Bedford, MA). Membranes were incubated with primary antibodies (Perforin, rabbit polyclonal; Granzyme, mouse monoclonal; IFN- γ , mouse monoclonal; all three from Santa Cruz Biotech, Santa Cruz, CA; actin, mouse monoclonal, Calbiochem, Billerica, CA) for 1 h at room temperature in 5% milk, 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1% Tween-20. Membranes were washed and incubated with secondary antibodies (anti-rabbit and anti-mouse) for 1 h and developed using ECL Western blotting system (Amersham Life Science, Arlington Heights, IL). After immunodetection, the protein positive bands were scanned and quantified using Scion image software. The ratio of the protein positive band to the actin band is presented in the text.

Statistical analysis The data shown in the figures and text are mean $\pm$ S.E.M. Data comparisons among multiple groups were made using one-way analysis of variance and the Student-Newman-Keuls test as a post-hoc test. A value of $p < 0.05$ was considered significant.

Results

We tested the effect of combined opiate agonist and antagonist treatment on pituitary tumor growth and immune function in fetal alcohol exposed female rats. We have previously shown that a smaller dose of μ opiate receptor antagonist, NALT, when combined with δ opioid receptor agonist, DPDPE, acts as a potent activator of NK cell cytolytic functions (Sarkar et al. 2012). In this study, we tested whether, by increasing the NK cytolytic function, the immune clearance of pituitary tumor cells can be achieved.

We used a well-established animal model of fetal alcohol exposure, in which female rats were exposed to alcohol (AF) or control diets (PF and AD) in utero and were used for study during adult period (Murugan et al. 2014; Gangisetty et al. 2015). When pituitaries of these animals were imaged by MRI around 3 month of age, image densities were light and their volume densities could not be accurately measured. The wet weight of pituitaries of these animals did not significantly differ between groups, although mean pituitary weight of AF rats was higher than PF and AD rats. (mg/pituitary; $n = 8$ /group; AF, 25.1 ± 7.3 ; PF, 16.0 ± 5.3 ; AD 17.7 ± 5.2 ; $F = 0.647$; $df 2, 21$, $p > 0.05$). However, female offspring of

Fig. 1 Changes in the growth of pituitary following estrogen treatment in fetal alcohol exposed animals. **a** Magnetic resonance imaging (MRI) pictures of rat pituitary (shown by circles) of representative AF, PF and AD rats treated with estrogen capsule for a period of 6 weeks. Changes in

the pituitary volume (**b**), pituitary weight (**c**) and plasma PRL levels (**d**) at the end of experimentation. Data are mean $\pm$ SEM of 8 independent experiments. *, $P < 0.05$, **, $P < 0.01$, different from PF and AD treated groups

Fig. 2 Effects of naltrexone and DPDPE on the growth of pituitary tumor following estrogen treatment in fetal alcohol exposed animals. **a** Magnetic resonance imaging (MRI) picture of rat pituitary (shown by circles) of representative AF, PF and AD rats prior to or two weeks after the placebo pellets (Control) and saline or naltrexone (NALT) pellets and DPDPE (NALT + DPDPE) treatments for 3 weeks. **b** Changes in the

mean pituitary volume pre and post opiodergic drugs treatments were shown. **c**, **d** Pituitary weight (**c**) and plasma PRL levels (**d**) at post treatment are shown. Data are mean $\pm$ SEM of 4–8 independent experiments. ^a, $P < 0.05$, compared to AD and PF of placebo-treated controls. ^b, $P < 0.05$, compared to similarly fed of placebo-treated controls

AF, AD and PF rats, after ovariectomy and implantation with an estradiol-17 β capsule, which elevates plasma estradiol level to 50 pg/ml (16), for six weeks show large and dense pituitary gland in MRI images (Fig. 1a; compare naïve vs AD, PF and AD pituitary images). Also, the pituitary volume density moderately changed between treatment groups (the mean value of AF group was higher than PF and AD groups, but

was not significantly different between groups; Fig. 1b). Determination of the wet weight of the pituitary and plasma level of PRL revealed that fetal alcohol exposed rat's pituitaries were bigger (Fig. 1c), and their plasma levels of PRL were higher than those in PF and AD groups (Fig. 1d). These data are consistent with previous findings (Gangisetty et al. 2015)) and suggest that fetal alcohol exposed rats develop more aggressive PRL-producing tumors than the control-fed animals.

The effect of opiate agonist and antagonist treatments on the growth of pituitary tumors was tested after treatments for a period of three weeks in estrogen treated AF, PF and AD rats. The growth of the pituitary tumor, prior to and after the opiate drugs treatments, was visualized using MRI and the percentage pituitary volume change was calculated. MRI images identified significantly more pituitary volume changes in vehicle treated (control) AF rats than the similarly treated PF and AD rats (Fig. 2a). Opiate agonist and antagonist treatment significantly reduced pituitary volume in AF rats and moderately, but not significantly, reduced pituitary volume in PF and AD rats. Pituitary weights and plasma PRL levels were compared between control-treated and opiate antagonist and

Fig. 3 Effects of naltrexone and DPDPE modulation of NK cell function in the spleen of fetal alcohol exposed rats. The levels of cytolytic regulatory factors (**a**: Granzyme, **b**: Perforin and **c**: IFN- γ) were determined in splenocytes obtained from spleens of AF, PF and AD rats implanted with placebo pellets (Control) or NALT pellets and DPDPE (NALT + DPDPE) for 3 weeks. Data are mean $\pm$ SEM of 8 independent experiments. ^a, $P < 0.001$, compared to AD, PF of placebo-treated controls. ^b, $P < 0.05$, compared to similarly fed of placebo-treated controls

Fig. 4 Schematic representation of a proposed mechanism by which naltrexone and DPDPE increases NK cells cytolytic functions to control the growth of prolactinomas in the pituitary. We propose that a combined treatment of MOR antagonist naltrexone and DOR agonist DAMGO enhances DOR activity to increase NK cell cytolytic functions and its tumor cell killing activity that results in suppression of the growth of prolactinomas in the pituitary gland

agonist-treated AF, PF and AD rats. Both pituitary weights and plasma PRL levels were significantly higher in AF rats than AD and PF rats in both control-treated and opiate antagonist and agonist-treated groups (Fig. 2c, d). NALT and DPDPE treatments significantly reduced the pituitary weight and plasma PRL level in both AF PF and AD animals.

Our previous studies indicated involvement of natural killer (NK) cells in the antitumor activity of opioid drugs (Boyadjieva et al. 2004, 2010; Sarkar et al. 2012). We, therefore, measured splenic cytokines levels in control-treated and opiate antagonist and agonist-treated AF, PF and AD rats. We found that granzyme (Fig. 3a), perforin (Fig. 3b) and IFN- γ (Fig. 3c), that are essential cytokines for NK cell function against cancer, are decreased in fetal alcohol exposed animals, and increased in NALT and DPDPE injected animals. These data suggest injection of the opiate drugs inhibited pituitary tumor growth in both normal and fetal alcohol exposed animals, possibly by increasing the NK cell cytolytic function (Fig. 4).

Discussion

There are several reports showing evidence for the existence of high levels of PRL in chronic alcoholic men and women. Alcohol-induced hyperprolactinemia was also reported in healthy, well-nourished women during residence on a clinical research ward for 35 days (Mello et al. 1988; Mendelson and Mello 1988). Alcohol-induced hyperprolactinemia has also been demonstrated in nonhuman primates (Mello et al. 1983) and laboratory animals (De et al. 2002). Studies conducted in macaque female monkeys showed that in some of these monkeys, the PRL levels were elevated after chronic self-administration of alcohol (3.4 g/kg/day) (Mello et al. 1983). Interestingly, immunocytochemical examination of the pituitary gland of one monkey showed apparent pituitary hyperplasia. We have recently obtained data showing that alcohol feeding during the adulthood increases plasma PRL levels and pituitary weight in cyclic female rats (De et al. 2002), and promote estrogen-induced development of prolactinomas in ovariectomized rats (De et al. 1995). We now extend this to show increased estrogen-induced pituitary volume, pituitary weight and plasma PRL levels in adult female rats exposed prenatally to ethanol. These data identify alcohol drinking might be an environmental factor promoting aggressive prolactinoma development in the pituitary.

Alcohol promotion of tumorigenesis in the pituitary may be due to multiple factors, including: i) defects in immune function, which leads to impaired immune surveillance; ii) abnormal hormone levels, such as stress hormones, which suppress immune function; and iii) altered organ development and gene expression in the affected tissue by developmental imprinting. We have focused on the effect of fetal alcohol

exposure on neuroendocrine regulation of the immune function. Fetal alcohol exposure lowers endogenous opioid function, increases body stress and reduces innate immune function (Kuhn and Sarkar 2008), and these pathological conditions changes are often correlated with higher incidences of cancers and infections (Bernstein et al. 2002; Pankov et al. 2002; Grinshpoon et al. 2005; Irwin and Miller 2007; Giovannucci and Michaud 2007; Giovannucci and Michaud 2007). Combined μ receptor antagonist and delta receptor agonist treatments have been found to increase peripheral NK cell activities and reduce breast tumor growth and metastasis. The data presented here show that similar opiate agonist and antagonist treatments were able to reduce pituitary tumor growth and hormone secretion in fetal alcohol exposed animals. These effects may possibly be due to the impact of opiate receptor regulated cytokine levels, such as increasing IFN- γ , perforin and granzyme B levels. These data provide a novel possibility in treating pituitary tumors and possibly other tumors using a combination therapy of NALT and DPDPE.

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