Histol Histopathol (1998) 13: 721-725

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Endothelin-1 enhances thymocyte proliferation in monolaterally adrenalectomized rats with contralateral adrenocortical regeneration

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Summary. Endothelins (ETs) are a family of vasoactive peptides widely distributed in the body systems, where they exert pleiotropic biological effects, acting through two main subtypes of receptors, named ETA and ETB. Evidence indicates that ET-1 plays a permissive role in the development of neural crest-derived structures, among which are the epithelial cells of the thymus. These cells are known to control proliferation and differentiation of thymocytes, a process requiring adequate levels of glucocorticoids. Therefore, we have investigated the effects of ET-1, that binds both ETA and ETB receptors, on thymocyte proliferation in monolaterally adrenalectomized rats with contralateral enucleated adrenal at day 4 and 8 of regeneration, when glucocorticoid production is very low and, respectively, rather normal. Metaphase index (percentage of metaphase arrested cells) of thymocytes is the lowest at day 4 of regeneration, and markedly rose at day 8, thereby confirming the need of sizable levels of circulating glucocorticoid for the maintenance of a normal rate of thymocyte proliferation. ET-1 markedly increased the mitotic index of thymocytes at both times of adrenal regeneration. At day 8 of regeneration, the ETA-receptor antagonist BQ-123 markedly lowered mitotic index of thymocytes, and annulled its ET-1-evoked raise. Conversely, the ETB-receptor antagonist BQ-788 was ineffective. Collectively, these findings clearly indicate that endogenous ETs, through the activation of ETA receptors, are involved in the maintenance and stimulation of thymocyte proliferation in the adult rat, thereby playing a possibly important role in the modulation of the immune-system functions.

Key words: Endothelin-1, Endothelin receptors, Thymus, Thymocyte proliferation, Rat

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Introduction

Endothelins (ETs) are a family of 21-amino acid vasoactive peptides, which are synthesized in and secreted by a wide variety of organs and systems, although their major source is vascular endothelium. Their pleiotropic autocrine/paracrine effects depend on the interaction with two main receptor subtypes, named ETA and ETB (for review, see Rubanyi and Polokoff, 1994; Ohlstein et al., 1996; Stojilkovic and Catt, 1996; Nussdorfer et al., 1997).

Despite the almost ubiquitous distribution of ETs in the body, only scarce data are available on their synthesis and action in the immune system. Monocyte/macrophages, but not T and B lymphocytes and neutrophils were found to synthesize ET-1 and ET-3 (Ehrenreich et al., 1990; Goto et al., 1996; Michael and Markewitz, 1996). Moreover, ET-1-like immunoreactivity and prepro-ET-1 mRNA have been detected in the spleen, bone marrow and lymph nodes (Hemsen and Lundberg, 1991; Sakurai et al., 1991). Studies dealing with the presence and the role of ETs in the functional regulation of the thymus in adult animals are completely lacking, although indirect proofs are available that ET-1 is involved in the thymus development (for review, see Kuwaki et al., 1997).

In the course of our current studies on the *in vivo* effects of ETs on the growth of normal and regenerating rat adrenal cortex (Malendowicz et al., 1997a-c; Markowska et al., 1997; Mazzocchi et al., 1997), we chose the thymus cortex to check the effectiveness and reliability of our methods of estimation of cell proliferation. Unexpectedly, we observed that ET-1, which binds both ETA and ETB receptors (Rubanyi and Polokoff, 1994; Ohlstein et al., 1996), enhances thymocyte proliferation in monolaterally adrenalectomized rats with contralateral enucleated gland undergoing regeneration. It therefore, seemed worthwhile to confirm this occasional finding and to ascertain the receptor subtype mediating this effect of ET-1.

Materials and methods

Animals and reagents

Adult female Wistar rats (160±10 g body weight) were kept under a 12:12 h light-dark cycle (illumination onset at 8:00 a.m.) at 23±1°C, and maintained on a standard diet and tap water *ad libitum*. ET-1, and the ETA- and ETB-receptor selective antagonists BQ-123 and BQ-788 (Ohlstein et al., 1996) were purchased from Neosystem Labs (Strasbourg, France), and vincristin from Gedeon-Richter (Budapest, Hungary).

Experimental procedures

Under ether anaesthesia, the left adrenal gland was enucleated and the contralateral gland removed. Operated rats were given 0.9% NaCl to drink, and were killed 4 or 8 days after surgery. Groups of rats (n=6) were given two subcutaneous (s.c.) injections of 2 nmol/kg ET-1, dissolved in 0.2 ml 0.9% NaCl, 24 and 12 h before the sacrifice. Other groups of animals (n=6) autopsied at day 8 of regeneration received two s.c. injections of 3 nmol/kg BQ-123 and BQ-788 alone or plus 2 nmol/kg ET-1. Control rats were injected with the saline vehicle. All groups of animals received an intraperitoneal injection of vincristin (0.1 mg/100g) 180 min before the autopsy. Rats were decapitated at 11:00 a.m.

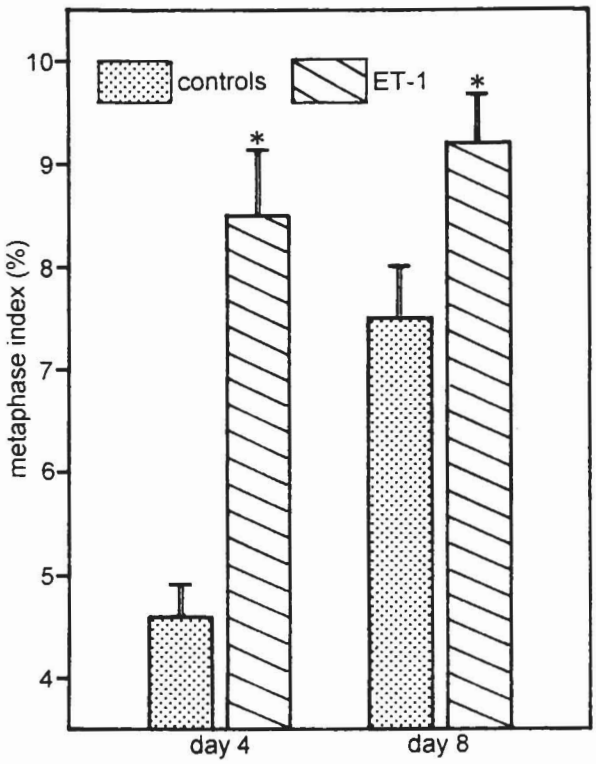

Fig. 1. Effects of ET-1 on rat thymocyte proliferation in the course of adrenal cortex regeneration (means±SEM; n=6). *: p<0.01 from the respective control group.

Thymocyte-proliferation assay

Thymuses were promptly removed, and capsule-adjacent fragments were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in Araldite. 0.5 μm-thick sections were cut and stained with toluidine blue. The mitotic index (% of metaphase-arrested cells) was calculated at x400, by counting 5,000 cells in the subcapsular zone (4-5 layers of cells) of each thymus cortex.

Statistical analysis

Individual results were averaged per experimental group, and SEM was calculated. The statistical comparison of the data was done by ANOVA, followed by the Multiple Range Test of Duncan.

Results

Metaphase index of thymocytes was the lowest at day 4 of regeneration, and significantly increased at day 8 (Fig. 1). ET-1 administration markedly raised mitotic index at both day 4 (60%) and day 8 (24%) (Figs. 1, 2). BQ-123 lowered the proliferation rate of thymocytes at day 8 (-20%) and prevented its ET-1-evoked rise, while BQ-788 was ineffective (Fig. 3).

Discussion

Our present findings provide clear-cut evidence that ET-1 enhances thymocyte proliferation in adult rats, through the activation of the ETA-receptor subtype. They appear to be in agreement with the results of previous studies showing that ETA receptors, probably by activating the MAPK cascade, stimulate the proliferation of various cell systems, including vascular smooth muscle cells (Weissberg et al., 1990; Zamora et al., 1993; Panettieri et al., 1996), Chinese hamster ovary cells transfected with the ETA gene (Sugawara et al., 1996), and rat adrenal zona glomerulosa cells (Belloni et al., 1996; Mazzocchi et al., 1997).

Compelling evidence suggests that both ET-1 and ETA receptors play a permissive role in the development of neural crest-derived craniofacial structures, among which the thymus. In mice with ETA receptor knockout, thymus is atrophic and its two anlages do not fuse at the midline (Kurihara et al., 1995; Kuwaki et al., 1997). The demonstration that BQ-123 *per se* evokes a sizeable reduction in the basal rate of thymocyte proliferation, allows us to suggest that endogenous ETs and ETA receptors play a major role in the physiological maintenance of the thymus growth not only in newborn, but also in adult rodents, thereby playing a possibly important role in the modulation of the immune-system

function.

Pluripotent cranial neural crest-cells migrate to the pharyngeal arches, where they differentiate into types I and II (among others) of thymic epithelial cells (Noden, 1991). Accordingly, these cells possess markers typical for neuroendocrine cells (Haynes et al., 1984; Ginda et al., 1996; Brelinska and Warchol, 1997; Dardenne and Savinio, 1997). Both types of thymic epithelial cells, and especially type II nurse cells, control thymocyte proliferation (Brelinska, 1989; Boyd et al., 1993), and accordingly kinetic studies show that about 95% of thymocyte proliferation takes place in the thymus cortex, mainly in the subcapsular zone which has to be considered the region where thymopoiesis is initiated (Scollay and Shortman, 1985; Boyd and Hugo, 1991; Scollay and Godfrey, 1995). There is general agreement that ETs are expressed in and modulate the function of neuroendocrine cells (for review, see Stojilkovic and Catt, 1996). This contention, along with the close spatial interrelationship between thymic epithelial cells and vascular endothelial cells in the outer zone of the thymus cortex (Sainte-Marie et al., 1986; Warchól et al., 1986), strongly suggests a paracrine/autocrine stimulatory action of locally produced ET-1 on the thymic epithelial cells, with the ensuing rise in the proliferation rate of thymocytes. In this connection, it is of interest to recall that thymic epithelial cells secrete interleukins (Lee et al., 1987), that several cytokines stimulate T-cell proliferation (Carding et al., 1991) and that ETs enhance cytokine secretion from various cell types (Michael and Markewitz, 1996).

Our study also shows that 8 days after adrenal enucleation the rate of thymocyte proliferation is higher that at day 4 of regeneration and does not significantly differ from that occurring in rats with intact adrenals (Malendowicz et al., 1997a). It is known that glucocorticoids in addition to stimulating lymphocytolysis, are also necessary for the intrathymic thymocyte survival and differentiation (Compton et al., 1987; Vacchio et al., 1994). We have previously shown that at day 8 of adrenocortical regeneration the level of circulating corticosterone is markedly higher than at day 4, and ET-1 enhances it at both time points (Malendowicz et al., 1997a,b). Evidence has been provided that in the thymus CRH and ACTH are expressed and a local glucocorticoid production occurs (Batanero et al., 1992; Arid

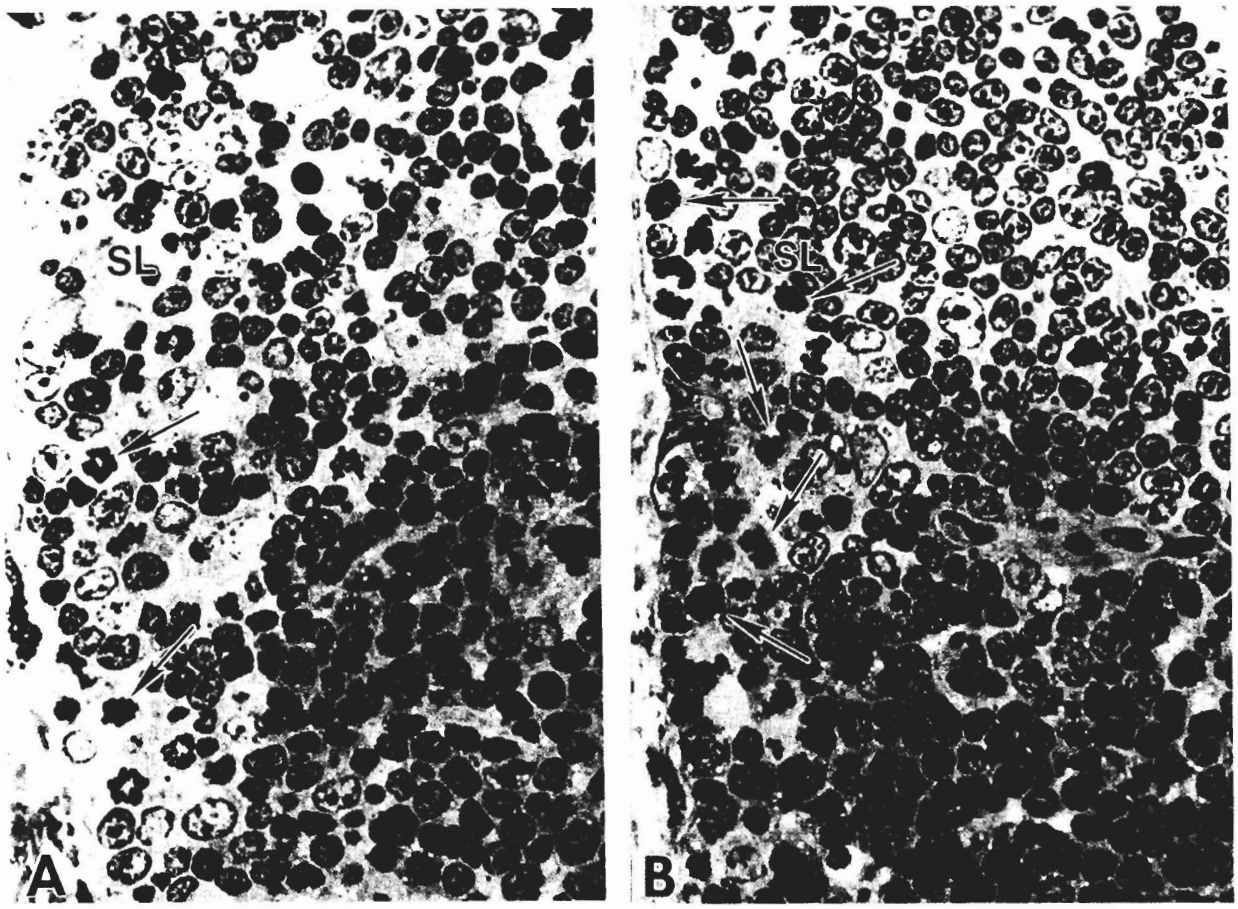

Fig. 2. Toluidine blue-stained 0.5 μm-thick sections of the thymus cortex at day 4 of adrenal cortex regeneration in control **(A)** and in ET-1-administered rat **(B)**. Mitoses (arrows) are visible in the subcapsular layers (SL), and ET-1 markedly increases their number. x 800

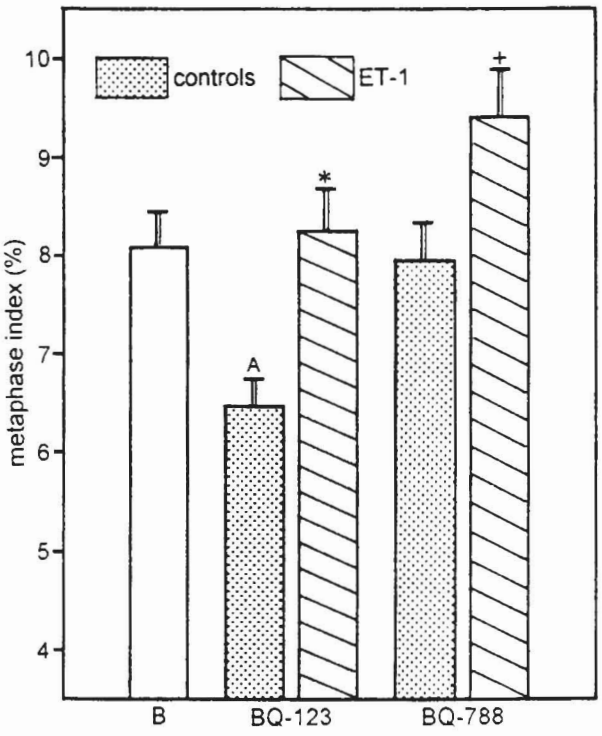

Fig. 3. Effects of ET-receptor antagonists on basal and ET-1-stimulated thymocyte proliferation in rats at day 8 of adrenal cortex regeneration (means SEM; n=6). +: $p<0.05$ and *: $p<0.01$ from the respective control group; A: $p<0.01$ from baseline (B).

et al., 1993; Vacchio et al., 1994; Dardenne and Savino, 1997). However, our above mentioned data appear to rule out the possibility that intrathymic steroidogenesis may be sufficient to maintain a normal thymocyte proliferation in the early stages of adrenal cortex regeneration, when glucocorticoid production is very low.

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Accepted January 5, 1998