Localization of Transforming Growth Factor ß1 (TGF ß1) on the Testis of Brown Banded-Bamboo Shark (*Chiloscyllium punctatum*)

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Summary

Growth factors are shown to promote cell proliferation and apoptosis, regulate tissue differentiation, and modulate organogenesis. In the present investigation, the Localization of transforming growth factor ß1 (TGF ß1) was studied on the testis of brown banded-bamboo shark by immune-histochemistry. The immunehistochemical reaction was present in the somatic cells, mainly Leydig cells, in most of testis zones in summer season, while in winter season the expression was confined to the Leydig cells of the spermatocytes, spermatids and spermatozoal zones. The reaction in the germ cells was clear in the spermatogonia of the germinal zone in the summer season while in winter it was clear in the head of sperms in the spermatozoal zone and negative in other zones in both seasons. In conclusion, these results demonstrate seasonal variation in the expression of TGFβ1 in the testes of this shark. This growth factor has a role in the differentiation and migration of spermatogonia, steroidgenesis through its effect on the Leydig cells either stimulating or inhibiting and finally apoptotic effect on either germinal epithelium or Leydig cells.

Key words

Transforming growth factor (TGFß1), testis, brown banded-bamboo shark.

Introduction

The testis of the brown banded-bamboo shark is divided into several lobes. Each lobe shows zonal arrangement and contained numerous round- shaped spermatocysts (follicles). These zones are germinal, spermatogonial, spermatocytes, spermatids, spermatozoal and degenerative zones (Kassab et al. 2009).

TGF-β family was implicated in the regulation of different biological processes including proliferation, differentiation, development and extracellular matrix production (Massague, 1998). The TGF-β super family entails more than thirty proteins sharing 30-80 % sequence homology (Ingman and Robertson, 2002). Five isoforms of TGF-βs (1-5) have been characterized (Ingman and Robertson, 2002), only three of them, TGF-β1, TGF-β2, and TGF-β3 have been identified in the mammalian tissues including testis (Teerds and Dorrington, 1993; Gautier et al., 1994; Lui et al., 2003). TGF-βs isoforms have been identified in the foetal, immature and adult testis of rat (Mullaney and Skinner, 1993; Teerds

and Dorrington, 1993; Gautier et al., 1994; Olaso et al., 1997; Jung et al., 2004) and pig (Avallet et al., 1994; Caussanel et al., 1997) as well as in the adult normal and varicocelized testis of human (Dobashi et al., 2002). These isoforms were shown to localize Sertoli, Leydig, peritubular, and germ cells (Mullaney and Skinner, 1993; Teerds and Dorrington, 1993; Gautier et al., 1994; Olaso et al., 1997; Jung et al., 2004; Avallet et al., 1994; Caussanel et al., 1997; Dobashi et al., 2002). Using immunohistochemistry, this investigation moreover, showed that all TGF-βs protein isoforms are expressed primarily in the interstitial cells. In adult rat testis, the most pronounced changes of TGF-βs immunoreactivity were displayed during spermatogenesis. High level of TGF-β1 in germ cells was observed at stages VIII and IX of the cycle and largely associated with spermatocytes and early spermatids but it declined rapidly thereafter to become eventually undetectable at stage X-VII (Teerds and Dorrington, 1993; Gautier et al., 1994).

The available literatures lack data about the effect of the growth factors on the testes of shark, so the aim of this work focused on the detection of the transforming growth factor ß1 (TGF ß1) on the testes of brown banded-bamboo shark.

Materials and Methods

Tissue Preparation

Four brown-banded bamboo male sharks were used in this study. Two were used at winter season (December-January) and two at summer season (June- August). The sharks were collected from Okinawa Churaumi Aquarium, Japan. The sharks ranged about 100 cm length and 4 kg weight. The sharks were dissected at the middle of body cavity, eviscerated and the testes were exposed. The length of the testes was measured. Cross section samples of the testes were fixed in bouin's solution for 18-24 hours at room temperature. The samples were extensively washed in 70% ethanol. Then after, the samples were dehydrated in graded series of ethanol (80%, 90%, 95% and absolute), cleared in lemosol and embedded in paraffin wax. Sections 3-5 microns thick were mounted on coated slides with 3-aminopropyl-triethoxysilane.

Immunohistochemical staining

For the detection of TGFß1, a mouse monoclonal antibody against TGF ß1 (Sigma, Tokyo, Japan) was used. Antigen localization was achieved using the avidin-biotin complex (ABC) technique (Hsu et al. 1981). Briefly, sections of paraffin embedded testicular tissue were dewaxed, rehydrated, and rinsed in PBS pH 7.4 (3×5 min). Endo-genous peroxidase was blocked by soaking the sections in 3% v/v hydro-gen peroxide/distilled water for 30 min at room temperature followed by washing them under running tap water for additional 10 min. Subsequently the slides were equilibrated in PBS pH 7.4 (2×5min). Non-specific antibody binding was minimized by covering the slides with bovine serum albumin (DAKO, Tokyo, Japan) for 30 min at room temperature. Sections were then incubated overnight at 4 C° with primary antibody against TGFß1 diluted 1:200 in antibody diluent (DAKO, Tokyo, Japan). The slides were subsequently rinsed in PBS pH 7.4 (2×5 min) followed by incubation with biotinylated rabbit anti-mouse IgG (diluted1:300 in PBS) for 30 min at room temperature.

Bounding antibodies were visualized using ABC kit and diaminobenzidine (DAKO, Tokyo, Japan). All incubations

were performed in a humidified chamber. Sections were left counter-stained in Mayer's haematoxylin, dehydrated, and mounted with Mount-Quick (Daido Sangyo co., Japan). Negative controls were performed by omission of the primary antibody.

Results

The expression of the transforming growth factor ß1 (TGF ß1) on testes was differed according to seasons, and also testicular zones (table1).

The germinal zone contained spermatogonia and immature interstitial Leydig cells in random distribution. The localization of TGF ß1 was restricted to the cytoplasm of spermatogonia in the summer season and not expressed in the spermatogonia of the winter season. The immature Leydig and Sertoli cells were negative in both seasons (Fig. 1, panels A and B).

The spermatogonial zone was characterized by the formation of the spermatocysts (follicles) from spermatogonia and Sertoli cells. The Leydig cells were occuping the inter spermatocysts part. The TGF ß1 was immunelabelled only to the Leydig cells of the shark testis in the summer season and not expressed in the same cell of the winter season. The spermatogonia and Sertoli cells were negative in both seasons (Fig. 1, panels C and D).

The reaction was restricted only to the Leydig cells in both summer and winter seasons in the spermatocytes and spermatids zones, while the spermatocysts with its content either spermatocytes or spermatids and Sertoli cells were not labeled (Fig.2, panels A, B and C).

The spermatozoal zone was like the previous zone in structure. The spermatocysts of the summer season was negative in reaction either in the sperms or the Sertoli cells, while the Leydig cells were reacted to the antibody. In the winter this zone differed in that, the strong reaction was to the head of the sperms and the weak reaction to the Leydig cells (Fig. 2, panel D and Fig. 3, panel A).

The degenerative zone showed evacuated cysts and Leydig cells in between. The cysts were negative to the antibody on both seasons. The Leydig cells were reacted to the antibody on the summer season but not on the winter season except in some cells (Fig. 3, panels B, C and D).

Table 1 : the immunohistochemical reaction of different zones of the testis

Testis zones	contents	summer	winter
Germinal zone	Spermatogonia	++ve	-ve
	Undifferentiated cells	-ve	-ve
Spermatogonial zone	Spermatogonia	-ve	-ve
	Sertoli cells	-ve	-ve
	Leydig cells	++ve	-ve
Spermatocytes zone	Spermatocytes	-ve	-ve
	Sertoli cells	-ve	-ve
	Leydig cells	++ve	++ve
Spermatids zone	Round spermatids	-ve	-ve
	Sertoli cells	-ve	-ve
		++ve	+ve
Sperma-	Sperm head	-ve	++ve

tozoal zone	Sertoli cells	-ve	-ve
	Leydig cells	++ve	+ve
Leydig cells	Evacuated cysts	-ve	-ve
	Leydig cells	++ve	-ve

Discussion

In the present study, the localization of transforming growth factor ß1 (TGF ß1) in the testis of brown banded-bamboo shark was investigated in the two seasons; summer and winter. The immunexpression is present in the somatic cells, mainly Leydig cells, in most of zones in the summer season, while in winter the expression is confined to the Leydig cells of the spermatocytes, spermatids and spermatozoal zones. The reaction in the germ cells is clear in the spermatogonia of the germinal zone in the summer and the head of sperms in the spermatozoal zone in the winter and negative in other zones in both seasons.

The presence of immunoreactivity in the spermatogonia of the summer season could be attributed to the TGF-βs proteins which play a variety of important roles in testicular development and function (Jung et al., 2004, Lui et al., 2003). In the mouse embryo and during genital ridge formation, TGF-β1 was shown to have chemotrophic effects on primordial germ cells migration (Godin and Wylie, 1991). In foetal testis, the expression of TGF-β1 and TGF-β2 at the time of seminiferous cord formation has led to the speculation that TGF-βs could play a role in this process (Olaso et al., 1997). These results have been confirmed by another fact that inhibitory effects of retinoid on the seminiferous cord formation and testis growth are mediated, at least in part, via an increase in the TGF-βs expression (Cupp et al., 1999). In addition, TGF-β1 inhibited epidermal growth factor (EGF)-stimulated testis growth, suggesting that TGF-β1 regulates embryonic development through other locally produced growth factors (Cupp et al., 1999). The above mentioned interpretations could be confirmed by the present results in the migration of spermatogonia in the germinal zone to form the round-shaped spermatocysts in the spermatogonial zone in the summer season. The immunlabelled head of the sperms in the spermatozoal zone in the winter season and not in the summer could be attributed to the apoptotic effect of the TGFβ1 and correspond with reports demonstrating a functional correlation with apoptotic process of TGFβ1 (Ohta etal.1996, Olaso et al. 1998 and Wagnar et al. 2005)

The presence of immunolabelled imamture Leydig cells in the spermatogonial zone might attributed to, TGF-β1 and TGF-β2 which could stimulate the production of seminiferous tubules basement membrane proteins ,moreover, the TGF-βs has the ability to stimulate the synthesis of the extracellular matrix component and to inhibit their degradation by numerous cell types (Massague, 1990).

The different expression courses throughout the year and different localization in somatic and spermatogenic cells in the seasonally breeding roe deer could attributed to both their involvement in the regulation of seasonal breeding and their most likely diverse function (Wagner et al., 2005). This explains the reactivity of spermatogonia in summer and not in winter season and also the head of the sperms in winter and not in summer.

The present study revealed the immunereactivity of the Leydig cells in all zones of the summer season and only the spermatocyte, spermatid and spermatozoal zones of the winter season. Dickson et al. (2002) have reported that TGF-β participated in the morphological differentiation of imamture Leydig cells into adult Leydig cells in the rat testis by inducing the expression of extracellular matrix protein, whereas TGF-β caused significant morphological changes in Leydig cells, which are accompanied by significant increases in secretion of fibronectin, laminin and collagen type IV and rearrangement of actin filaments in TGF-β-treated cells. Conversely, Geiger et al. (1999) demonstrated that TGF-β inhibits Leydig cell proliferation in the pig and TGF-β therefore, may be responsible the cessation of the Leydig cell proliferation at the end of each wave of Leydig cell development and may inhibit the cells from dividing prematurely in the immature testis. This explains the immunoreactivity of the Leydig cells of the degenerative zone in the summer seasons which may indicate the seasonal breeding of the shark.

TGF-β1 and TGF-β2 could be autocrine factors involved in the decrease of testosterone production by foetal Leydig cells during late foetal life (Olaso et al., 1997). TGF-β1 is known to be potent inhibitor of differentiated functions of pig (Avallet et al., 1994) rat (Lin et al., 1987), and mouse (Bebakar, et al., 1990) Leydig cells from immature and adult testes by decreasing LH receptors number, LH/hCG stimulated cAMP and testosterone production. Additionally, both TGF-β1 and TGF-β2 were shown to decrease LH-stimulated testosterone production by rat foetal Leydig cells in vitro (Gautier et al., 1997).

In conclusion, these results demonstrate seasonal variation in the expression of TGFβ1 in the testes of this shark species. This growth factor has a role in the differentiation and migration of spermatogonia, steroidgenesis through either stimulating or inhibiting effects on the Leydig cells and finally apoptotic effect on either germinal epithelium or Leydig cells.

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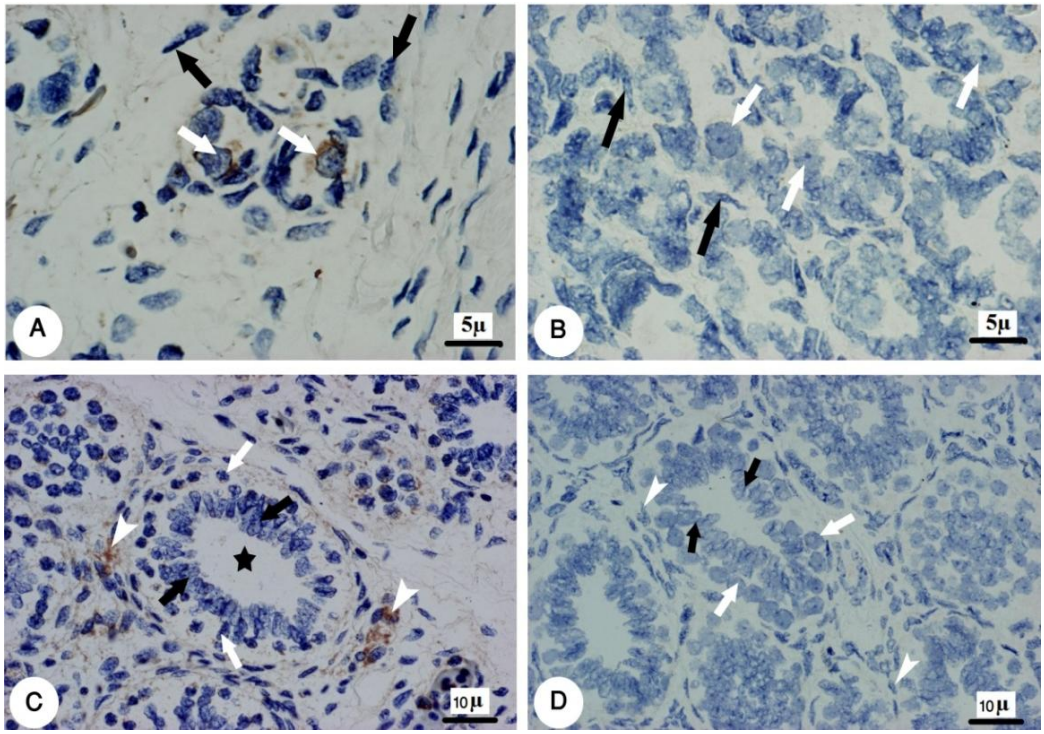

Fig (1)

Panel A, photomicrograph of the germinal zone of the testis of the brown banded-bamboo shark in the summer season showing immunlabelled spermatogonia (white arrow) and negative undifferentiated Leydig cell (black arrow) bar 5µ.

Panel B, the same zone in the winter season showing, negative reaction to both spermatogonia (white arrow) and undifferentiated Leydig cell (black arrow), bar 5µ.

Panel C, spermatogonial zone in the shark testis of the summer season showing, negative spermatogonia (white arrow) and Sertoli cell (black arrow) in the spermatocyst (black asterisk). The immunlabelling was restricted to the undifferentiated Leydig cells (white arrow head), bar 10µ.

Panel D, the spermatognial zone in the winter season showing, negative to the spermatogonia (white arrow), Sertoli cell (black arrow) and undifferentiated Leydig cells (white arrow head), bar 10µ.

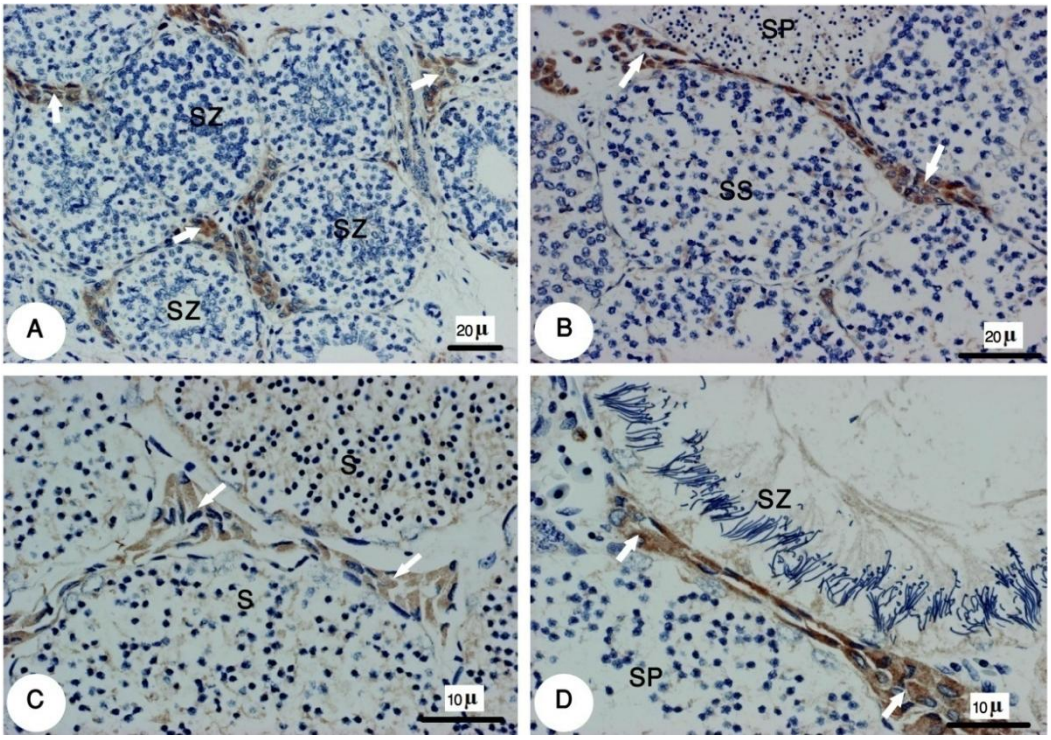

Fig (2)

Panel A, spermatocyte zone of the testis of the shark in the summer season showing, negative spermatocysts (SZ) and immunlabelling Leydig cells (white arrow), bar 20µ.

Panel B, the junction between the spermatocyte and spermatid zones in the shark testis of the summer season showing, immunlabelld Leydig cells (white arrow) and negative to spermatocyte spermatocysts (SS) and spermatid spermatocysts (SP), bar 20µ.

Panel C, Spermatocyte zone of the testis of the shark in the winter season showing, weak immunlabelling to the Leydig cells (white arrow) and not to all spermatocysts (S), bar 10µ.

Panel D, the junction between the spermatozoal and spermatid zones of the shark testis in the summer season showing immunlabelled Leydig cells (white arrow) and negative in the spermatozoal spermatocysts (SZ) and spermatid spermatocyst (SP), bar 10µ.

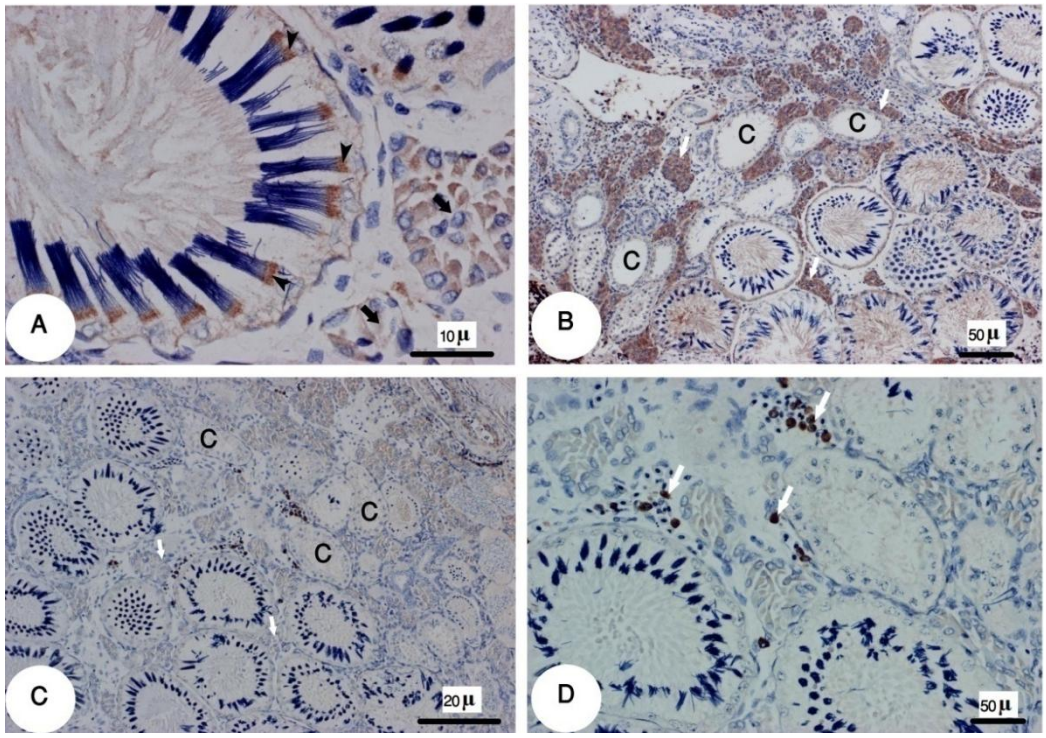

Fig (3)

Panel A, spermatozoal zone in the shark testis in the winter season showing, reacted head of the sperm (black arrow head), weak reaction to the Leydig cells (black arrow), bar 10µ.

Panel B, degenerative zone of the summer season showing, reacted Leydig cells (white arrow) and negative evacuated cyst (C), bar 50µ.

Panel C, degenerative zone of the winter season showing, negative Leydig cells (white arrow) and negative evacuated cyst (C), bar 20µ.

Panel D, degenerative zone of the summer season showing, reacted some cells between Leydig cells (white arrow), bar 50µ.