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Immunohistochemical pattern of insulin-like growth factor (IGF) I, IGF II, and IGF binding proteins 1 to 6 in carcinoma in situ of the testis

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Abstract

***Aim*—To study the immunohistochemical localisation of insulin-like growth factor (IGF) I, IGF II, and IGF binding proteins 1–6 in intratubular germ cell neoplasia in the vicinity of solid germ cell tumours of the testis.**

***Methods*—Testes were obtained from 13 patients (20–35 years old) who had undergone orchidectomy for treatment of a solid germ cell tumour. Tumour cells were verified histologically by their distinctive morphology and by visualisation of placental alkaline phosphatase immunoreactivity.**

***Results*—The majority of carcinoma in situ (CIS) cells were immunopositive for IGF I, whereas no CIS cells stained for IGF II. Of all the IGF binding proteins investigated, CIS cells showed intense immunoreactivity for IGF binding protein 5 and lower expression of all other IGF binding proteins.**

***Conclusions*—These results suggest that the action of IGF binding protein 5 in CIS cells may modulate the activity of IGF I. This may be related to a proliferative advantage that could facilitate tumour development.**

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Keywords: insulin-like growth factor; IGF binding proteins; germ cell tumour; carcinoma in situ; testis; tumour marker

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Carcinoma in situ (CIS) or testicular intraepithelial neoplasia[1–3] constitutes the preinvasive lesion of testicular germ cell tumours.[2 4–7] CIS cells are thought to be derived from fetal germ cells (gonocytes) and are considered to be the precursors of all testicular germ cell tumours arising in adults.[2] This implies that these cells are already present at birth and that genetic and endocrine factors during puberty trigger the transition from CIS cells to solid germ cell tumours.[8] The hypothesis that adult germ cell tumours develop from polyploid CIS[9] is supported by the observation that the DNA index in CIS cells is close to that of seminoma.[10] An increase in hyperdiploid cell frequency has been described in the seminal fluid of patients with CIS associated with or without a frank tumour.[11] However, a reliable early diagnosis of germ cell tumours requires the detection of CIS cells in surgical biopsies. This is of particular importance in the case of patients with a testicular tumour who have a significant risk of developing malignancy in the contralateral testis.[4]

Frequently, seminiferous tubules adjacent to solid germ cell tumours are occupied by Sertoli cells that surround CIS cells and residual spermatogonia. CIS cells can be detected by immunohistochemistry for placental alkaline phosphatase[1] and are characterised morphologically by large, round nuclei with huge nucleoli, cytoplasmatic glycogen, mitochondria with widened cristae, and the relative paucity of electron dense substance between neighbouring organelles.[12]

Insulin-like growth factors (IGF) I and II are growth promoting factors that act via autocrine and paracrine mechanisms in the testis. Their effect is mediated predominantly through the type I IGF receptor. In addition, six IGF binding proteins have been identified and these play a crucial role in determining the in vivo action of IGF I and IGF II.[13] To date, the IGF system has not been investigated systematically in early germ cell tumours and, therefore, we studied the pattern of distribution of IGFs and IGF binding proteins in CIS cells by immunohistochemistry.

Methods

TISSUE PREPARATION

Testes were obtained from 13 patients, aged 20–35 years, who had undergone orchidectomy for a solid germ cell tumour. Small pieces of tissue about 0.5 cm thick adjacent to the solid tumour were fixed in Bouin's solution for 24 hours at room temperature and, after dehydration in increasing alcohol concentrations, embedded in paraffin wax.[12] The tissue investigated (figs 1 and 2) was preselected such that it had seminiferous tubules with numerous tumour cells, accompanied exclusively by Sertoli cells to avoid misinterpretations resulting from residual germ cells. The presence of tumour cells was verified histologically by their distinctive morphology and confirmed by visualisation of placental alkaline phosphatase immunoreactivity.[1 14–17] In addition, four cases showed seminiferous tubules with morphologically intact spermatogenetic epithelium. The intertubular tissue exhibited groups of Leydig cells and occasional lymphocyte infiltration.

IMMUNOHISTOCHEMISTRY

For the immunocytochemical studies, 6 μm thick paraffin wax sections were mounted on to chromalaun-gelatin coated slides and incubated with primary antibodies for 24 hours at 4°C in a humified chamber. Monoclonal

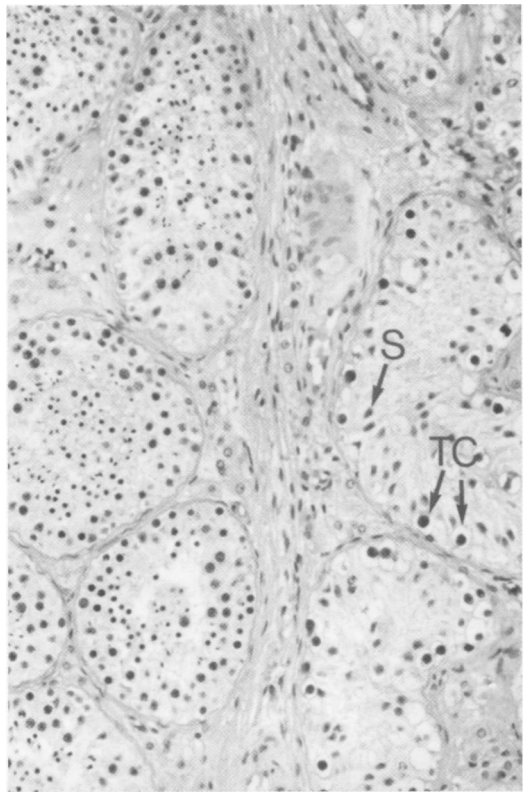

Figure 1 Survey of testicular tissue adjacent to a solid germ cell tumour showing two testicular lobules separated by a connective tissue septum. Left lobule: seminiferous tubules exhibiting active spermatogenetic epithelium. Right lobule: tubules containing exclusively tumour cells (TC) and Sertoli cells (S). (Paraffin wax section; haematoxylin and eosin; original magnification ×160.)

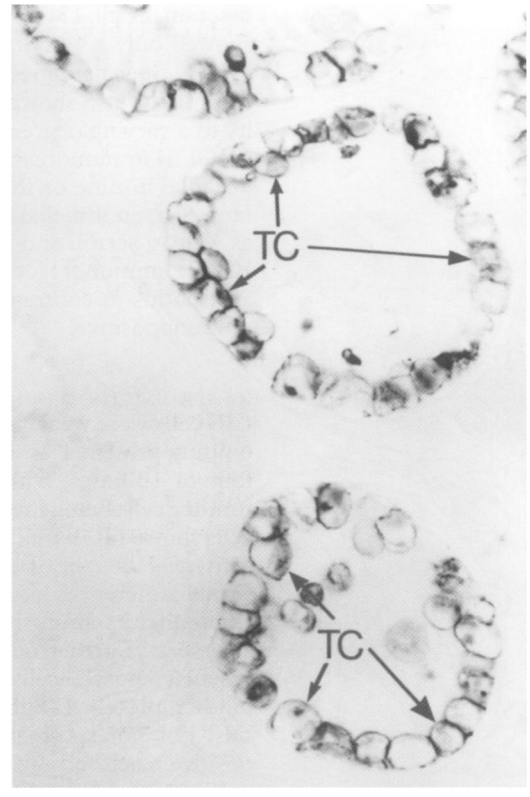

Figure 2 Verification of tumour cells showing placental alkaline phosphatase immunoreactivity. TC, tumour cells. (Original magnification ×330.)

mouse antibodies directed against human IGF I (final dilution 1/100 (vol/vol) in phosphate buffered saline (PBS) with 0.1% (wt/vol) sodium azide and 0.2% (wt/vol) radioimmunoassay grade bovine serum albumin) and IGF II (1/40 (vol/vol)) (Serotec, Oxford, UK) were used. Polyclonal rabbit antibodies against human IGF binding protein 1 (1:750 (vol/vol)), 3, 4, and 5 (1:500 each (vol/vol)), bovine antihuman IGF binding protein 2 (1/1000 (vol/vol)) (Upstate Biotechnology, Lake Placid, New York, USA), and bovine antihuman IGF binding protein 6 (1/50 to 1/100 (vol/vol)) (Austral Biologicals, San Ramon, California, USA) were also used. Visualisation of the antigens was performed by means of a combination of peroxidase antiperoxidase and avidin-biotin-peroxidase complex (ABC) methods.[18] This technique enhances the staining intensity and allows the visualisation of small amounts of antigen. In the second step, biotinylated mouse or rabbit IgG (Dako, Hamburg, Germany) diluted 1/250 (vol/vol) was applied for 60 minutes at room temperature. This step was followed by a 30 minute incubation with mouse or rabbit peroxidase antiperoxidase diluted 1/100 or 1/200 (vol/vol), respectively, and an ABC complex (Vector Laboratories, Burlingame, California, USA) diluted 1/250 (vol/vol) for 30 minutes at room temperature. The peroxidase activity was developed by means of the nickel-glucose oxidase approach as described by Záborszky and Léránt.[19]

NEGATIVE CONTROLS

Three negative controls were set up: the primary, secondary, and tertiary antibodies were replaced by PBS; only the peroxidase activity was visualised; or sections were incubated with normal rabbit or mouse serum (Sigma, Deisenhofen, Germany or Dako) instead of the primary antibodies, at concentrations ranging from 0.01% to 0.1% (vol/vol). In addition, preabsorption studies were carried out for IGF binding proteins 3 and 5 to evaluate the specificity of the reaction. Maximal inhibition of the reaction was achieved with 35 μg/ml of IGF binding protein 3, and 20 μg/ml of IGF binding protein 5 (fig 3).

CELL COUNTING

Frequently, CIS tubules contained 10–30 (or more) tumour cells per cross section, depending on the size of the tubule. In these cases, at least 100 tumour cells could be counted for each section investigated, allowing semiquantitative estimations of the percentages of expressing cells. If fewer tumour cells per section were present (this was the case in four samples), additional sections 20 μm distant from each other were used to count at least 100 tumour cells.

Results

IGF I AND IGF II IMMUNOREACTIVITY

In 11 cases, IGF I immunoreactivity was detected in tumour cells as a peripheral subplasmalemmal ring and/or paranuclear accumulation of the IGF I/anti-IGF I complex (fig 4A). However, cell counts showed that 46% (26–65%) of the tumour cells were immunopositive. Endothelial tissue, vascular muscle, and Sertoli cells showed a positive

reaction in all 13 cases, whereas Leydig cells showed only a weak reaction in nine of the 13 cases. Spermatogonia, spermatocytes, and spermatids also showed IGF I immunoreactivity to a varying degree.

IGF II immunoreactivity in tumour cells was negative in nine of the cases studied (fig 4B). However, endothelial and vascular muscle cells as well as Sertoli and Leydig cells all exhibited IGF II immunoreactivity. Spermatocytes and spermatids in contrast to spermatogonia were immunopositive.

IGF BINDING PROTEIN 1–6 IMMUNOREACTIVITY

CIS cells were weakly immunoreactive for IGF binding protein 1 as a paranuclear dot in only four of 13 cases, with 60% (53–68%) of the tumour cells being involved. Sertoli and Leydig cells showed IGF binding protein 1 immunoreactivity. However, Leydig cells showed individual differences of staining intensity and a pronounced staining of Reinke crystalloids was also seen. Furthermore, endothelial cells and vascular muscle cells were positive for IGF binding protein 1. Spermatocytes and spermatids, but not spermatogonia, showed a weak positive reaction.

In 12 of 13 cases, tumour cells were IGF binding protein 2 immunopositive and showed a paranuclear dot-like reaction (fig 4C). The staining was more intense than IGF binding protein 1. Forty five per cent (9–76%) of the tumour cells present were positive. Sertoli cells, Leydig cells, as endothelial cells, and muscle cells of the blood vessels were all positive for IGF binding protein 2. Spermatocytes and spermatids showed only a weak staining for IGF binding protein 2, whereas spermatogonia were negative.

IGF binding protein 3 immunoreactivity was present in 57% (41–78%) of the tumour cells. In nine of 13 cases they showed a weak reaction as a paranuclear dot. Sertoli and Leydig cells as well as endothelial cells and smooth muscle cells of the blood vessels preserved IGF binding protein 3 immunoreactivity. Spermatogonia, spermatocytes, and spermatids showed nuclear localisation of the reaction product in addition to that in the cytoplasm.

Weak IGF binding protein 4 immunoreactivity was present in only one case and seen as a paranuclear dot. About 20% of the tumour cells were positive for IGF binding protein 4. Sertoli cells were weakly immunopositive in six of 13 cases and Leydig cells showed very strong immunoreactivity with individual differences in staining intensity. Pronounced staining of Reinke crystalloids in the Leydig cells were also evident. Endothelial cells and smooth muscle cells of the vasculature showed a weak reaction in 10 of 13 cases. Spermatocytes and spermatids showed a positive reaction, stronger than with IGF binding protein 2, whereas spermatogonia were negative.

IGF binding protein 5 immunoreactivity was present in tumour cells of all cases, seen as a peripheral subplasmalemmal ring and/or as a paranuclear dot. In three cases, this was also seen in a proportion of the nuclei (fig 4D). IGF binding protein 5 immunoreactivity was the most intense of all the IGF binding proteins investigated and all tumour cells examined were intensely positive. Sertoli cells were negative. In Leydig cells, a moderate immunoreactivity was

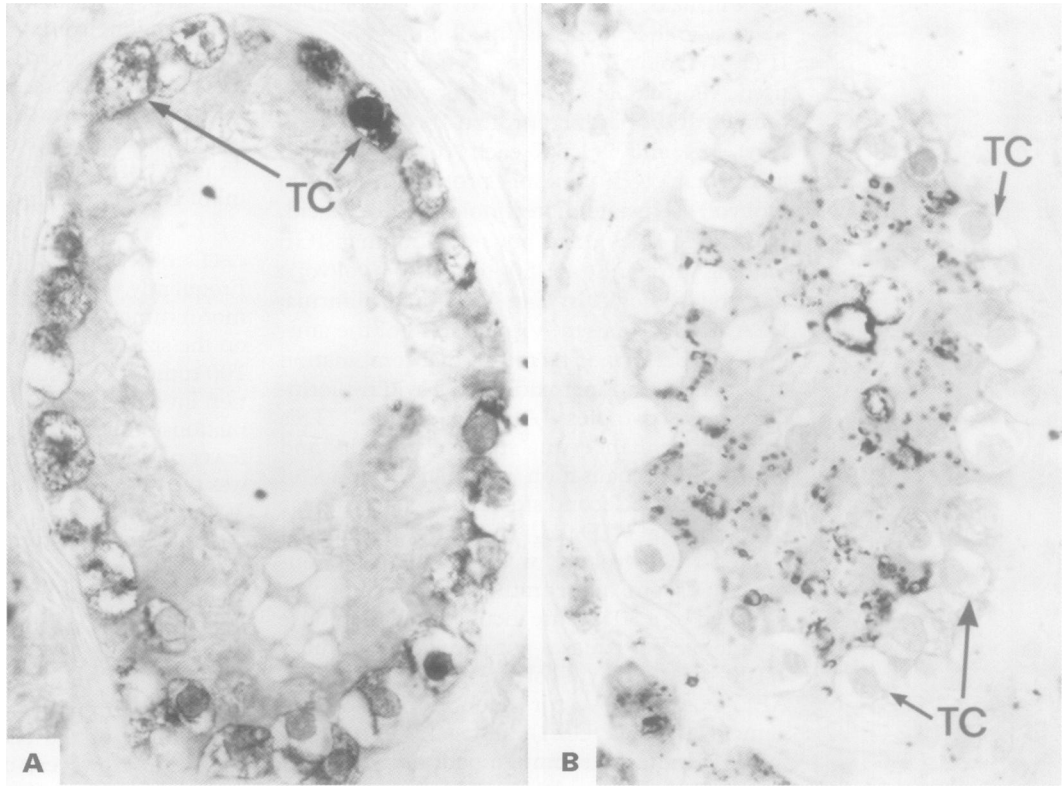

Figure 3 Preabsorption studies. (A) IGF binding protein 5 immunoreactivity in tumour cells (TC). (B) The reaction is abolished by preincubation with 20 μg/ml of the corresponding antigen. In this incubation, non-specific depositions appear in non-tumour cells. (Original magnification ×520.)

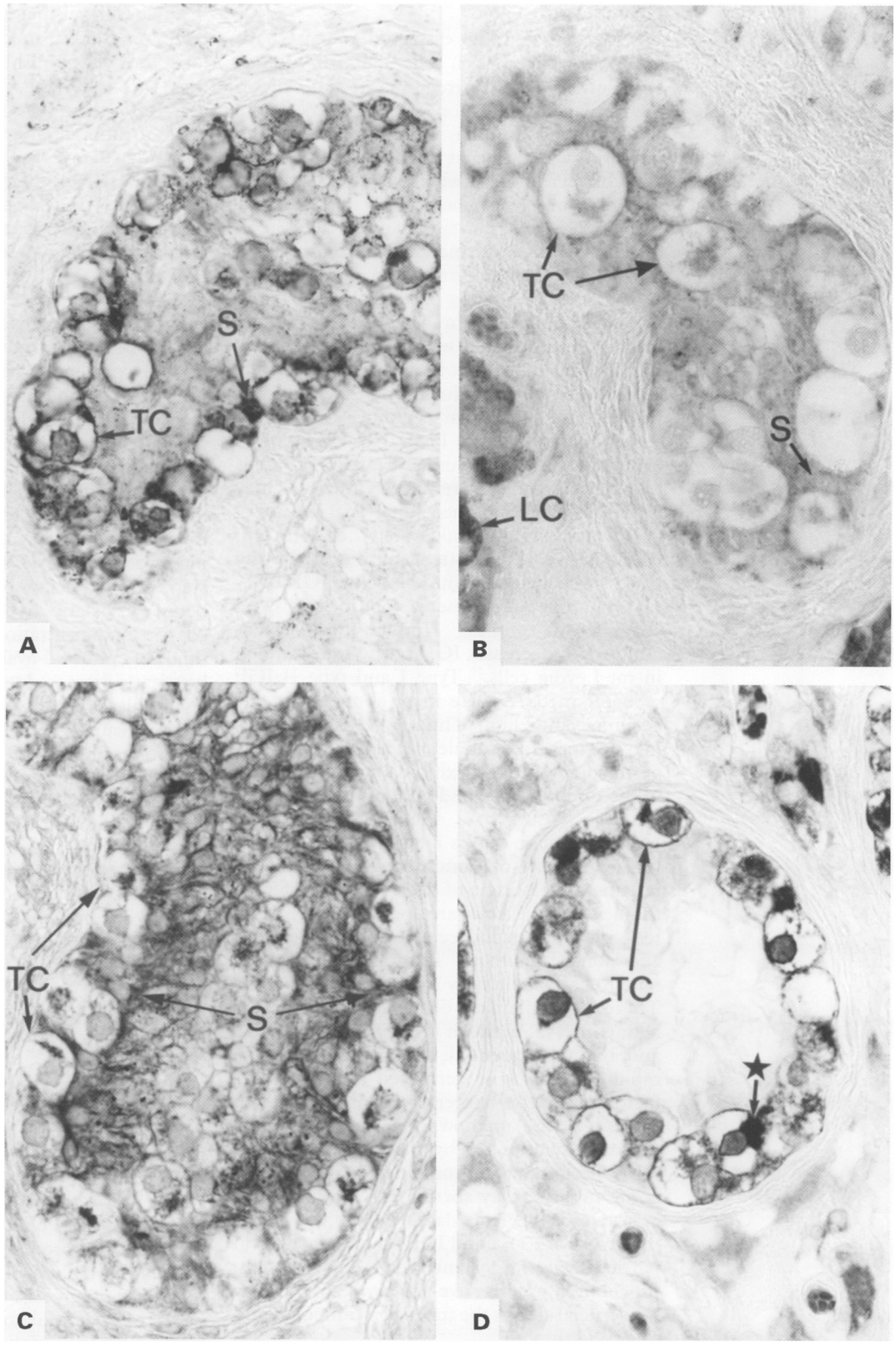

Figure 4 Testicular tubules containing exclusively tumour cells (TC). (A) IGF I immunoreactivity in tumour cells. Distinct staining is also present in Sertoli cells. (B) No IGF II immunoreactivity in tumour cells. Moderate staining of Sertoli and interstitial Leydig cells (LC). (C) IGF binding protein 2 immunoreactivity in tumour cells and Sertoli cells. (D) Intense IGF binding protein 5 immunoreactivity in tumour cells, seen as peripheral rings and/or as a paranuclear dot (asterix). (Original magnification ×520.)

observed, with variation in staining intensity and pronounced staining of Reinke crystalloids. Endothelial cells and vascular muscle cells showed a weak reaction in eight cases. Spermatogonia, spermatocytes, and spermatids showed a strong reaction. This was predominantly cytoplasmic, but some spermatogonia showed nuclear staining.

Only weak IGF binding protein 6 immunoreactivity could be detected in tumour cells in four of 13 of the cases, seen as a paranuclear dot in 41% (29–61%) of the tumour cells. Sertoli cells, Leydig cells, endothelial cells, and vascular muscle cells showed immunoreactivity, whereas no reaction was seen in spermatogonia, spermatocytes, or spermatids.

Discussion

We have studied the distribution pattern of immunoreactive IGF I, IGF II, and IGF binding proteins 1–6 in carcinoma in situ cells of the human testis. Among all members of the IGF binding protein family, IGF binding protein 5 was the most abundant in CIS cells and, furthermore, it was strongly positive in spermatogonia, spermatocytes, and spermatids. IGF binding protein 5 was detected in all tumour cells as confirmed by placental alkaline phosphatase immunoreactivity. In contrast, IGF I and IGF binding proteins 1, 2, 3, 4, and 6 were found only in a proportion of the tumour cells, whereas no CIS cells stained for IGF II. This may result from the state of differentiation of the individual tumour cells or may be because particular epitopes are more susceptible to fixation. The variable location of immunoreactivity within individual CIS cells (subplasmalemnal, paranuclear, nuclear) may reflect differences in their functional states.

Normal testis is a moderately rich source of IGF I, derived from both Sertoli[20 21] and Leydig cells.[22 23] Testicular immunoreactive IGF I was first found in culture media conditioned by rat seminiferous tubules.[24] Human chorionic gonadotropin decreases IGF I gene transcription in rat Leydig cells.[25] Type I and type II IGF receptor mRNAs were most abundant in the germinal epithelium of human testis.[26] Interestingly, cryptorchid tubules, which show a lack of germinal epithelium, exhibit a marked increase in the numbers of type I IGF receptors in Sertoli cells.[27]

Carcinoma in situ is identified by a characteristic pattern of tumour cells lying on the basement membrane of testicular seminiferous tubules.[2] These cells are found frequently in testicular tubules adjacent to solid seminomas and non-seminomas.[28] Intratubular CIS cells tend to have a relatively low mitotic activity.[15 29] This could explain the observation that there is a long interval between the appearance of CIS and the subsequent development of a solid, rapidly proliferating germ cell tumour.[30] The factors crucial for triggering the proliferation of these cells are unknown. However, they are probably synthesised by the CIS cells themselves because, when contained in the seminiferous tubules, they survive and proliferate in culture for several days.[31] Our data may indicate that IGF I and IGF binding factor 5 in particular are important factors for CIS cells, providing a proliferative advantage which may be a crucial step in the transition from CIS cells to a solid tumour.

Autocrine regulation of growth occurs in a number of tumours, suggesting that tumour cells can respond to the same growth factor that they synthesise.[32] This has been well described in testicular tumours. Two out of three seminomas were shown to express IGF II mRNA transcripts, embryonal carcinoma being the richest source of IGF II mRNA, whereas a teratoma did not express IGF II transcripts.[33] An increase in IGF II concentrations has been found in two seminomas and one teratoma out of five testicular tumours (two seminomas, three teratomas) examined, the highest levels being found in a seminoma.[34] Seminomas were moderately immunoreactive for IGF II (unpublished data), which is in contrast to our findings in CIS cells, suggesting that IGF II production may depend on their state of differentiation. Human pluripotential teratoma (Tera-2) cells are dependent on IGF I or IGF II for their survival in serum free medium.[34–36] These cells, when deprived of serum, undergo apoptosis which can be counteracted by simultaneous addition of IGF I or IGF II.[34 35]

A similar immunocytochemical study of the early stages of germ cell tumours (CIS cells) has not been performed previously. The results of the present study suggest a possible functional significance of the IGF–IGF binding protein system. For example, in high grade prostatic intraepithelial neoplasia, IGF binding protein 2 immunostaining intensity was enhanced significantly in comparison with normal epithelium; in addition, it was increased even further in malignant cells[37] and IGF binding protein 5 mRNA was overexpressed in prostatic stromal cells isolated from patients with benign prostatic hyperplasia.[38] IGF binding protein 2 concentrations were measured in cyst fluids of epithelial ovarian cancer and found to be greatly increased in malignant cysts, regardless of histological type.[39] IGF binding protein 2, as measured by both western ligand blotting and immunoblotting, was significantly higher in malignant than in benign epithelial ovarian neoplasms. These findings were supported further by immunohistochemical detection of IGF binding protein 2 in tumour sections.[40] In addition, concentrations of IGF binding protein 2 were found to be raised in serum from patients with nephroblastoma and this molecule has been proposed as a tumour marker.[41]

Depending on the particular cell system, IGF binding proteins modulate the action of IGFs either in an inhibitory or stimulatory fashion. Extracellular matrix contains IGF binding protein 5, which potentiates IGF I action, suggesting that the binding of IGFs to the matrix, via IGF binding protein 5, may be important in eliciting the cellular growth response to these growth factors.[42] In contrast, IGF binding protein 4 inhibits IGF I mediated effects on Leydig cells.[43] Furthermore, we have observed that glioma cell lines rarely express IGF binding protein 4 mRNA transcripts,[44] whereas normal Leydig cells show high levels of IGF binding protein 4 expression.[43] Thus, the balance of stimulatory and inhibiting IGF binding proteins may be of pivotal importance for the proliferative capacity of both normal and malignant cells.

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