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EXPERIMENTAL STUDY

Development of an experimental ovarian tumor: immunocytochemical analysis

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Abstract

Objective: The aim of the present work was to study whether immunocytochemical parameters present in the normal ovary were altered after tumor development under high gonadotropin levels.
Methods: Ovarian tumors (luteoma): castrated female rats had an ovary grafted into the spleen; tumors were left to develop for 1, 2, 3 or 7 months. The presence of apoptotic cells (TUNEL method) and the expression of proliferating cell nuclear antigen (PCNA), gap junction protein (Cx43), steroidogenic acute regulatory protein (StAR), aromatase and synaptosome-associated protein of 25 kDa (SNAP-25) were determined by immunocytochemistry. Some of these findings were confirmed by RT-PCR (Cx43, StAR, SNAP-25). Inhibin subunit mRNAs were investigated by Northern blot.
Results: PCNA staining of tumors was mainly found in granulosa cells of transforming follicles and was absent from luteinized follicles. A nearly complete absence of apoptosis was observed. Cx43 was mainly found in follicles, while it was very weakly expressed or absent in luteinized follicles. StAR protein expression, indicating active steroidogenesis, was demonstrated only in luteinized follicles and in thecal cells, but was absent from granulosa cells. Aromatase immunoreactivity was very intense in granulosa and also present in luteal cells. Membrane-associated and cytoplasmic SNAP-25 immunostaining was determined in patches of endocrine cells in the follicles, as well as in the luteinized follicles. The expression of mRNAs for Cx43, StAR and SNAP-25 (RT-PCR) and inhibin subunits (Northern blots) were confirmed in 1-, 3- and 7-month-old tumors.
Conclusions: These results indicated that luteoma most likely develop from unruptured follicles by hypertrophy and proliferation of follicular cells. Circulating gonadotropins seem to play a fundamental role in maintaining the expression of proteins typically expressed in normal ovary, while avoiding apoptosis in this tissue.

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Introduction

An ovary implanted into the spleen of an ovariectomized rat develops into a tumor, growing in response to gonadotropins. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels are high in these animals as the steroids secreted by the tumor are poured into the hepatic-portal vein and are consequently metabolized by the liver before reaching the general circulation (1, 2). In this way the negative feedback, which is normally exerted at the hypothalamic and hypophyseal levels, is abolished. This tumor grows and acquires approximately three times the initial volume of the grafted estrous ovary after 2 months (3) and 20 times after 1 year of development (4). We have demonstrated that this tumor depends on gonadotropins for its growth by treating tumor-bearing rats with buserelin, a gonadotropin-releasing hormone (GnRH) agonist that downregulates LH and FSH secretion; this resulted in very significant tumor regression (3). A direct action of GnRH analogs on the tumor has also been demonstrated (5, 6). In women, the risk of suffering ovarian cancer has been related to gonadotropin levels. High levels of gonadotropins in the early postmenopause have been suggested to play a role in the development of ovarian neoplasms. This is supported by the dramatically increased incidence of ovarian cancer in women above the age of 45 years, when gonadotropins reach high levels (7, 8). Therefore, the dependency on gonadotropins for growth in this experimental ovarian tumor is similar to the one proposed for different

ovarian pathologies such as certain ovarian carcinomas (9, 10), granulosa cell tumors after ovarian stimulation in treatments of infertility (11) or in the polycystic ovary syndrome (12), rendering this tumor an interesting model for the study of tumor development under hypergonadotropinemia.

This experimental ovarian tumor is highly luteinized (luteoma) (4) and hormonally active since it secretes estradiol, progesterone and inhibins (4, 5). Inhibins have been proposed as markers of ovarian tumors (13, 14).

The aim of the present study was to further characterize this experimental ovarian tumor by determining different immunocytochemical parameters described in normal ovary (15–20), to evaluate if it was maintained or modified during tumor development under high gonadotropin levels.

For this purpose, the expression of proliferating cell nuclear antigen (PCNA) and the presence of apoptotic cells by the TUNEL method were investigated. In addition, parameters related to tumor secretion capacity, such as the expression of steroidogenic acute regulatory protein (StAR), a key protein involved in steroid biosynthesis, aromatase, the enzyme which converts androgens into estrogens, the synaptosome-associated protein of 25 kDa (SNAP-25), this last one being involved in exocytotic processes, and connexin 43 (Cx43), which participates in ovarian cell interaction, were also determined. Where possible, these findings (Cx43, StAR, SNAP-25) were confirmed by RT-PCR in 1-, 3- and 7-month-old tumors. The expression of inhibin subunit mRNAs was analyzed by Northern blot in these tumors at the same stages of development.

Materials and methods

Animals

Adult female virgin Sprague–Dawley rats (200–250 g) from the Instituto de Biología y Medicina Experimental colony were housed in groups in an air-conditioned room, with lights on from 0700 to 1900 h. They were given free access to laboratory chow and tap water. At the end of experimental procedures, animals were killed by decapitation according to protocols for animal use approved by the institutional animal care and use committee (IBYME-CONICET) which follows NIH guidelines. Animals were cycled daily and, after two regular cycles, they were operated on during the morning of estrus. Surgical procedures were performed as previously described (2). Briefly, animals were anesthetized with ketamine (100 mg/kg body weight i.p.), both ovaries were removed and one gonad was cleared of the adherent fat and oviduct and was inserted into the spleen. Tumors were left to develop for 1, 2, 3 or 7 months. Thereafter, animals were decapitated and the tumors were either embedded in paraffin for immunocytochemical analysis or completely cleared from adherent tissue and homogeneized in TRIZOL (Gibco-BRL, Rockville, MD, USA) for RNA extraction.

Immunocytochemistry

Tissue sections were deparaffinized and hydrated as described by Fritz *et al.* (18). The cellular distribution of PCNA, Cx43 and SNAP-25 in the tumors was determined by immunohistochemistry, using commercially available monoclonal or polyclonal antisera: anti-PCNA (1:100) (Calbiochem, Bad Soden, Germany), anti SNAP-25 (1:500) (Sternberger monoclonals Inc., Baltimore, MD, USA) and anti rat-Cx43 (1:500) (Sigma, Deisenhofen, Germany) (16). In addition, a well-characterized StAR antibody (1:1000) (19) and an aromatase antiserum (1:500–1:2000) (21) were used. Immunohistochemical procedures using the avidin–biotin method were employed as described previously (18). Immunoreactivity was visualized with diaminobenzide. For control purposes, the first antiserum/antibody was omitted and incubations with normal rabbit serum/mouse serum were carried out instead. Sections were examined with a Zeiss Axiovert microscope.

For detection of apoptotic cells, the *In Situ* Cell Death Detection Kit (Boehringer Mannheim, Mannheim, Germany) was employed and procedures were followed as indicated in the manufacturer's instructions.

The complete sets of immunocytochemical experiments were performed on five different 1- or 2-month-old tumors.

RT-PCR analyses and sequencing

Total RNA was prepared from 1-, 3- and 7-month-old tumors by the Chomczynski & Sacchi method (22) which utilizes the TRIZOL reagent. Normal 3-month-old rat ovary and normal rat spleen were used as controls. Total RNA (100–500 ng) were used for reverse transcription utilizing an 18-mer polydeoxythymidine primer and Moloney's murine leukemia virus reverse transcriptase (Promega, Mannheim, Germany). Amplification of Cx43, StAR and SNAP-25 was performed as described (16–19, 23). In order to be able to compare RT-PCR reactions of different animals, only those RNA samples were used which, after RT and PCR using tubulin primers, yielded comparable bands. PCR amplification consisted of 30–35 cycles of denaturing (94 °C, 15 s), annealing (55 °C, 1 min) and extension (72 °C, 2 min). The PCR reaction products were separated on 2% agarose gels and visualized with ethidium bromide. They were verified by sequencing, as described (16–19, 23).

Oligonucleotide primers used for amplification of rat Cx43, α-tubulin, rat StAR and rat SNAP-25 were synthesized according to the published GenBank

sequences, namely Cx43 GenBank (accession number X06656); sense: 5′-GCGGCGGCTTCACTTTC-ATTA-3′ corresponding to nt 158–179; antisense 5′-CAGACGTTTTCGCAGCCAGGTTG-3′ complementary to nt 371–395; tubulin (accession number K 00558); sense: 5′-CACCCGTCTTCAGGGCTTCTTGGTTT-3′ corresponding to nt 398–415; antisense: 5′-CATTTCAC-CATCTGGTTGGCTGGCTC-3′ complementary to nt 779–796; StAR (accession number RNU76419); sense: 5′-TGGAGAGGCTCTATGAAGAGC-3′ corresponding to nt 987–1004; antisense: 5′-GCCACGTAAGTT-TGGTCTTAG-3′ complementary to nt 1214–1233; primer design in comparison with Ronen-Fuhrmann *et al.* (24); SNAP-25 (accession number AB003991); sense: 5′-ATGGCCGAGGACGCAGACATGCGTAAT-3′ corresponding to nt 1–27; antisense: 5′-AGCATCACT-GGATTTAAG-3′ complementary to nt 283–300.

Northern blot determination of inhibin subunit mRNAs

Total RNA from 1-, 3- or 7-month-old tumors and estrous ovaries (four to five samples per group) was extracted, as described above. Northern blots were performed as described by Woodruff *et al.* (25). Briefly, 20 μg total RNA, previously verified for integrity, was subjected to horizontal electrophoresis in denaturing agarose–formaldehyde gels and transferred to nylon membranes (Hybond-N; Amersham Life Science, Little Chalfont, Bucks, UK). Membranes were exposed overnight at 65 °C to heat-denaturalized probes for α, βA, βB inhibin subunits (generously provided by Dr Aaron Hsueh) or glyceraldehyde 3-phosphate dehydrogenase (G3PDH) as an internal control, labeled with ^{32}P (Random Primed DNA Labeling Kit; Boehringer Mannheim). Membranes were then exposed to X-ray films (Biomax MS; Eastman Kodak Company, Rochester, NY, USA). Molecular sizes were estimated with RNA molecular weight standards (Promega).

Results

Tumor development

Cell proliferation, detected by an antibody recognizing PCNA, was mainly found in transforming follicles and was absent from luteinized follicles (Fig. 1). A major difference from normal ovaries was the almost complete absence of apopotic cells after 1–2 months of tumor development, as determined by the TUNEL method (Fig. 2).

A prominent feature indicating local interaction of ovarian cells, namely the presence of gap junctions consisting of Cx43, was also demonstrated in tumor samples (Fig. 3). Interestingly, Cx43 was mainly found in follicles (granulosa cells) but not in luteinized follicles, which thus differs from active corpora lutea in the normal ovary (15).

Tumor secretion capacity

A immunocytochemical analysis of hormone production capacity of 1-month-old tumors was undertaken by determining the presence of key enzymes and factors involved in hormone synthesis or secretion. Active steroidogenesis is indicated by high StAR protein expression. StAR was demonstrated only in luteinized follicles and in thecal cells, but was absent from granulosa cells of follicles, indicating that the latter do not participate in *de novo* steroid production (Fig. 4).

Aromatase, the enzyme which coverts androgens into estrogens, was very intense in granulosa cells and also present in luteal cells (Fig. 5), in agreement with high estradiol output by tumor cells (5).

SNAP-25, which is a marker for exocytotic processes, was found in patches of endocrine cells in the follicles, as well as in luteinized follicles (Fig. 6, left). In line with previous observations (17), membrane-associated and cytoplasmic staining was observed. Neither SNAP-25 nor tyrosine-hydroxylase (TH)-immunoreactive nerve

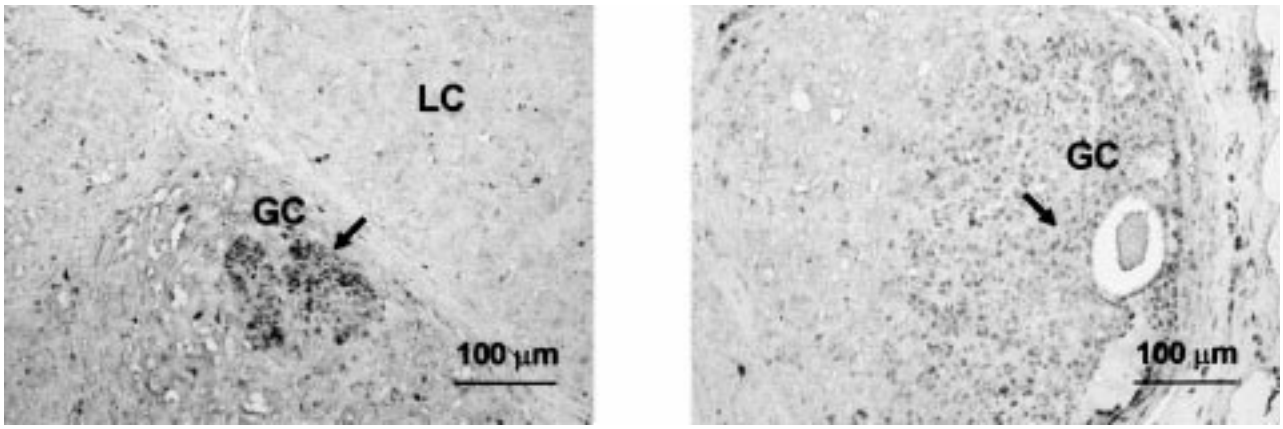

Figure 1 PCNA immunoreactivity in 1-month-old luteoma samples. Anti-PCNA antibody was diluted 1:100. Immunostaining was observed mainly in granulosa cells. Arrows indicate positive anti-PCNA staining. GC: granulosa cells and LC: luteal cells.

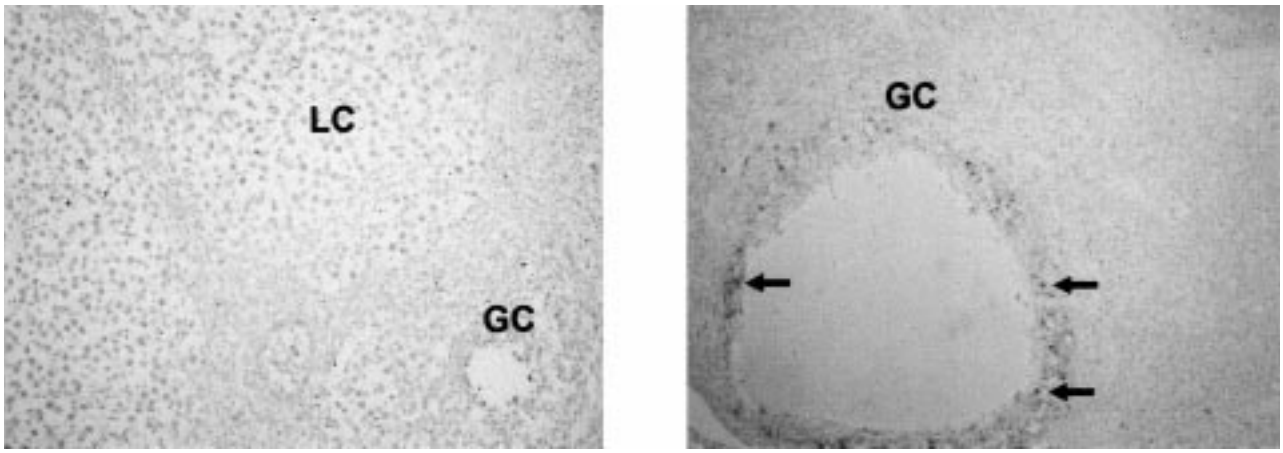

Figure 2 Apoptotic cell staining by the TUNEL method in 1-month-old luteoma sections (left panel). Note the absence of staining in luteoma cells when compared with a control ovary section (right panel, positive cells in large antral follicle indicated by arrows). GC: granulosa cells and LC: luteal cells.

fibers were found in the transplants, but were present in the spleen (TH staining not shown) (Fig. 6, right).

A summary of the expression of the ovarian markers in the different cell types of the experimental ovarian tumors is shown in Table 1.

Table 1 Immunocytochemical expression of ovarian markers in experimental ovarian tumors.

Ovarian marker	Granulosa cells	Luteal cells	Thecal cells
PCNA	++	−	−
Cx43	++	−	−
StAR	−	++	+
Aromatase	+++	+	−
SNAP-25	+	+	−

RT-PCR and Northern blot analyses

RT-PCR analysis confirmed the presence of mRNA for rat (r) Cx43, rStAR and rSNAP-25 in 1- and 3-month-old luteoma, indicating that they are present in the tumors at different stages of development and when a high degree of luteinization has taken place (Fig. 7). Furthermore, rCx43, rStAR and rSNAP-25 expression were also present in 7-month-old tumors (not shown).

Expression of mRNA for inhibin subunits α (1.4 kb), βA (5.8 and 3.2 kb) and βB (3.5 kb) was detected by Northern blot in 1-, 3- and 7-month-old tumors. The G3PDH mRNA (1.2 kb), expressed constitutively, was used as an internal control (Fig. 8). Characteristic patterns of expression were evident for each of the inhibin subunits; while α expression remained fairly constant during tumor development, βA and βB were highly expressed at 1 month of development and decreased to nearly undetectable levels at 3 months of development.

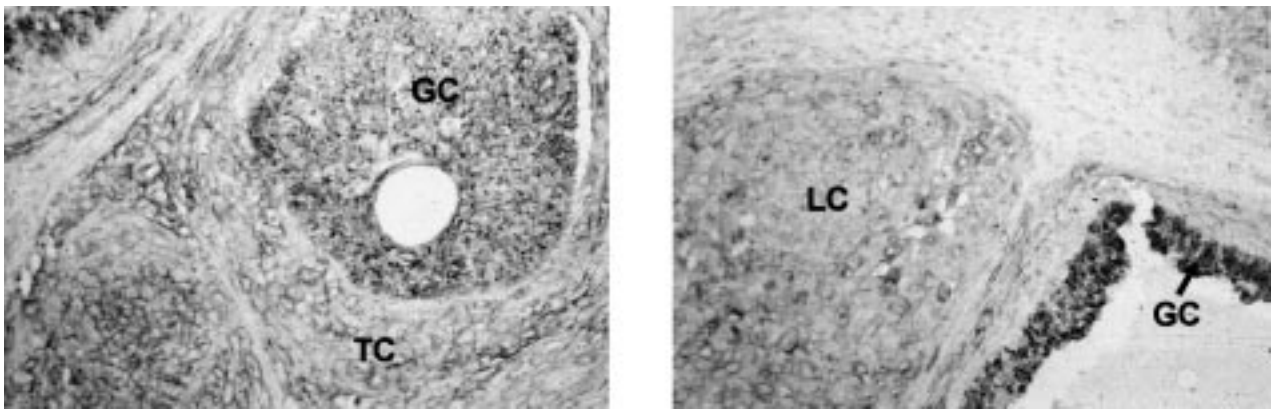

Figure 3 Cx43 immunoreactivity is demonstrated in 1-month-old luteoma sections using a specific Cx43-antiserum (1:500). Note positive staining mainly in granulosa cells (arrow). GC: granulosa cells, LC: luteal cells and TC: thecal cells.

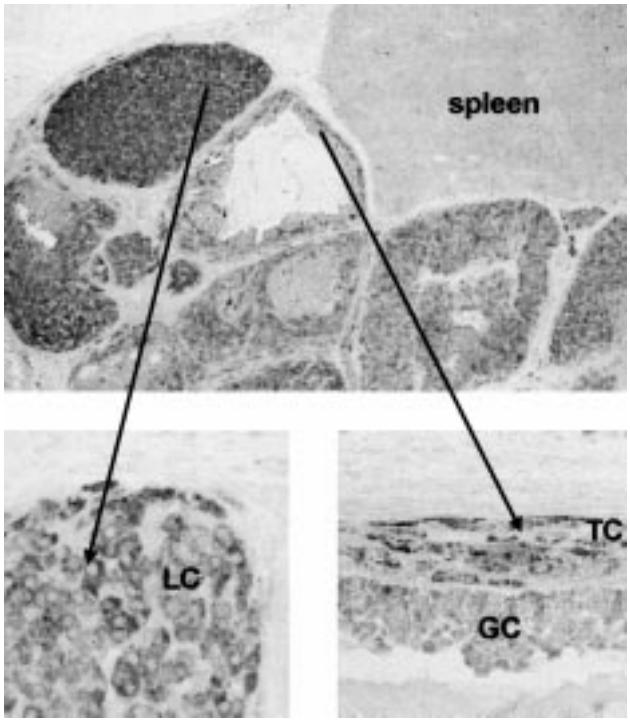

Figure 4 StAR protein immunostaining in 1-month-old tumor sections (antibody 1:1000). Positive staining was demonstrated only in luteinized follicles and in thecal cells, but was absent from granulosa cells of follicles. GC: granulosa cells, LC: luteal cells and TC: thecal cells.

Discussion

The aim of the present study was to further characterize this experimental intrasplenic ovarian tumor by determining various immunocytochemical parameters described in the normal ovary, in order to establish if these suffered any modification during tumor development under constant gonadotropin hyperstimulation.

Effective growth of a certain tissue is the result of the balance between proliferation and cell death. Both parameters were examined in these intrasplenic ovarian tumors. Cell proliferation (PCNA-positive cells) was mainly found in the granulosa cells of transforming follicles and was absent from luteinized follicles, in agreement with data in the literature (26). These data suggest that luteoma tumors most likely develop from unruptured follicles by hypertrophy and proliferation of follicular cells, under the influence of high gonadotropins present in luteoma-bearing animals, in agreement with data in the literature for growing follicles (27, 28), although the participation of other proliferating agents (29–31) secreted by this tumor (5, 32) cannot be discarded. The low index of proliferating cells in luteoma tissue is in agreement with the benign characteristics of this tumor (4), as high PCNA indexes in ovarian tumors have been related to malignancy and poor prognosis (33). Apoptosis, on the other hand, is a normal cellular process involved in various events of cell turnover in the ovary, such as follicle atresia or corpus luteum demise (34). In the luteoma, a major difference with regard to normal ovaries was the almost complete absence of apopotic cells after 1–2 months of tumor development, as determined by the TUNEL method. This observation suggested the hindrance of corpora lutea regression or of follicle atresia in this experimental luteoma, in which both corpora lutea and follicles in different stages of development and luteinization were observed. Both gonadotropins have been described as rescuing ovarian cells from apoptosis (34–36). Therefore, the high circulating gonadotropins present in luteoma-bearing animals may be responsible for the low apoptotic index observed. Together, these data on proliferation and

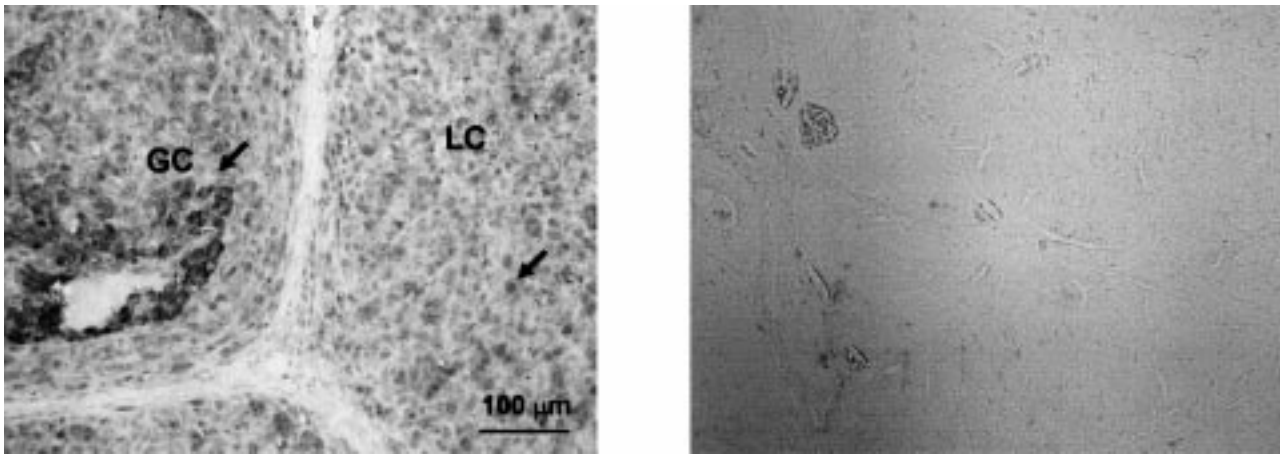

Figure 5 Aromatase immunoreactivity (antibody 1:500) in 1-month-old tumor sections was very intense in granulosa cells and also present in luteal cells (left panel). Note the total absence of staining when tissue was incubated without primary antibody (right panel). GC: granulosa cells and LC: luteal cells.

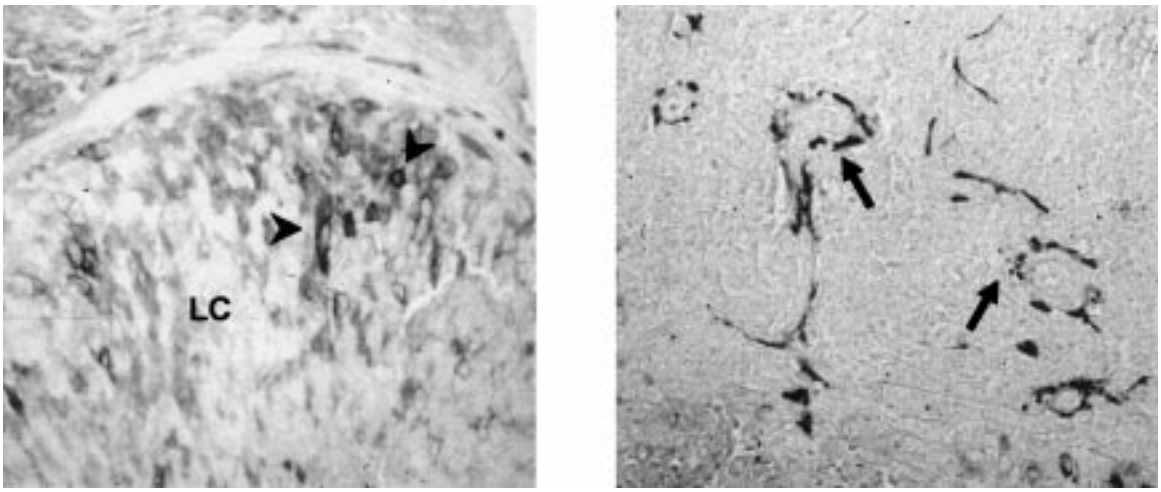

Figure 6 SNAP-25 immunoreactivity (antibody 1:500) in 1-month-old luteoma sections (left panel) and spleen sections (right panel). Membrane-associated and cytoplasmic staining was found in patches of endocrine cells in luteinized follicles (arrow heads), as well as in developing follicles (not shown). SNAP-25 immunoreactive nerve fibers were present in the spleen but absent from transplants (arrows). LC: luteal cells.

apoptosis suggest that the luteoma represents a highly differentiated non-metastatic tumor model, which most likely develops from unruptured follicles by hypertrophy and proliferation of granulosa cells and persistence of corpora lutea. In addition, our results do not support the idea that ovarian cancer can be solely attributed to high circulating gonadotropin levels, as has been suggested. Although gonadotropins may mediate ovarian hyperplasia and hypertrophia in the luteoma model, this input does not appear to induce malignant transformation of ovarian cells, at least in the period assessed in this study. This conclusion is supported by the work of Kumar *et al.* (37, 38) in transgenic models, where they propose that gonadotropins are modifier factors for gonadal tumor development in inhibin-deficient mice.

A characteristic marker of ovarian cells is the expression of the protein Cx43 present in gap junctions. Gap junctions show a particular distribution pattern during follicle development and subsequent corpus luteum formation and demise (15, 39–41). It has been demonstrated that FSH increases levels of Cx43 mRNA and its phosphorylation, changes which are associated with reduced proliferation and enhanced differentiation (16, 42). On the other hand, LH induced, as an immediate response, the phosphorylation

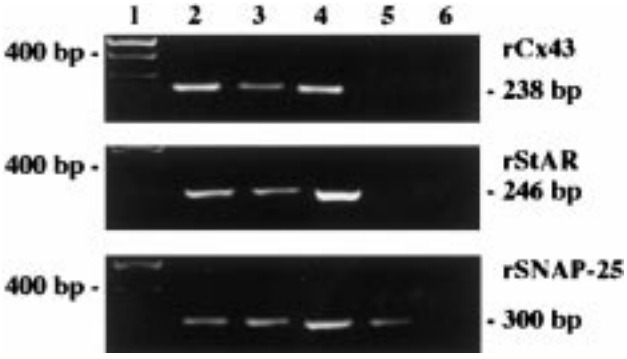

Figure 7 Expression of rCx43, rStAR and rSNAP-25 in luteoma cells by RT-PCR using specific primers synthesized according to the published GenBank sequences. (1) 100 bp DNA ladder, (2) 1-month old luteoma, (3) 3-month-old luteoma, (4) normal 3-month-old rat ovary, (5) normal rat spleen and (6) control PCR reaction, omission of template. Note the absence of rCx43 and rStAR expression in the spleen and the presence of SNAP-25 in agreement with immunocytochemistry results showing immunoreactive nerve fibers in the spleen.

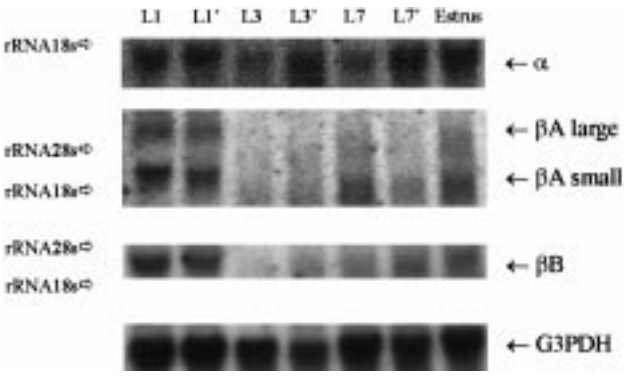

Figure 8 Expression of inhibin subunit mRNAs in 1-, 3- and 7-month-old luteoma by Northern blot (L1, L3 and L7 and L1′, L3′ and L7′ respectively; two independent luteomas in each case). Estrous ovaries were used as controls (Estrus). α (1.4 kb), βA (large: 5.8 kb and small: 3.2 kb) and βB (3.5 kb) subunits were detected. The G3PDH mRNA (1.2 kb), expressed constitutively, was used as an internal control. Arrows on the left indicate the migration of 18S and 28S rRNAs.

of Cx43 and, as a later response, the reduction of Cx43 protein concentration, because of attenuation of its gene expression (43). Interestingly, loss or dysfunction of gap junctions appears to be important in allowing cancer cells to escape growth regulation, as has been shown by the loss of Cx43 expression in human ovarian carcinoma cells (44). In consequence, Cx43 is an interesting factor to analyze in luteoma sections. In these tumors, Cx43 was mainly found in follicles (granulosa cells) but was very weakly expressed in or absent from luteinized follicles; this therefore differs from active corpora lutea in the normal ovary. The presence of Cx43 was confirmed by RT-PCR in 1-, 3- and 7-month-old luteoma, indicating that this gene is still expressed after prolonged tumor development. The loss of Cx43 expression in luteinized follicles may be due to persistently high LH, as this hormone has been shown to downregulate the expression of Cx43 (42, 43) and luteinized tissue is more responsive to LH than to FSH. Therefore, although corpora lutea do not degenerate in this tumor (see above), they seem to lose some of the traits of active luteal tissue. In this regard, we have previously shown that luteoma cells secrete less progesterone and more estradiol than other highly luteinized cells (5).

StAR protein is the hormone-stimulated factor responsible for transfer of cholesterol from cellular stores to the inner mitochondrial membrane and is thus the true rate-limiting step in steroidogenesis; its activation by gonadotropins is critical for progesterone production by the corpus luteum (19, 45). In rats bearing intrasplenic ovarian autotransplants, peripheral steroid levels are not elevated, due to shunt of blood flow to the liver, where they are metabolized (3). However, active steroidogenesis is indicated by high StAR protein expression, in agreement with progesterone and estradiol secretion into the splenic vein, which collects tumor output (3). StAR was demonstrated only in luteinized follicles and in thecal cells, but was absent from granulosa cells of follicles, indicating that the latter do not participate in *de novo* steroid production, in agreement with previous data (24, 46). The presence of StAR mRNA was confirmed by RT-PCR in luteoma of 1, 3 and 7 months of development, suggesting active steroidogenesis even in late stages of tumor development and that the expression of this protein is probably maintained by high persistent gonadotropin levels present in these animals.

The key enzyme catalyzing the last step of estradiol biosynthesis in the ovary is P_{450} aromatase. Its expression and regulation in rat granulosa cells has been found to be associated with follicular development and luteinization (47, 48) and this enzyme is also frequently present in benign and malignant ovarian tumors (49). In luteoma, aromatase expression was very intense in granulosa cells and was also present in luteal cells, in agreement with high estradiol output by tumor cells (5). As with other factors described above, we suggest that high gonadotropins maintain aromatase expression in luteoma cells, in agreement with that which has been described in the normal ovary (50, 51).

The synaptosome-associated 25 kDa protein was initially described in the nervous system (52) and it was demonstrated to be involved in regulated exocytosis (53). Grosse *et al.* (17) recently demonstrated its expression in non-neuronal, non-neuroendocrine compartments of the ovary, specifically in steroid-producing cells such as follicular and luteal cells, as well as in oocytes. Ovarian cells, in addition to steroids, also synthesize many other products, including peptides, growth factors and hormones, which are secreted by regulated exocytosis, and this process may involve SNAP-25. In luteoma sections, we determined membrane-associated and cytoplasmic SNAP-25 immunostaining in patches of endocrine cells in follicles, as well as in luteinized follicles, in agreement with our previous observations (17). It is noteworthy that neither SNAP-25 nor TH-immunoreactive nerve fibers were found in the transplants (but were present in the spleen), indicating that, after transplantation into the spleen, the ovaries do not became readily re-innervated. Elevated FSH in luteoma-bearing animals may be involved in the regulation of SNAP-25 expression in luteoma cells, as was demonstrated in the GFSHR-17 granulosa cell line (17). In addition, the presence of SNAP-25 mRNA was confirmed by RT-PCR during tumor development.

Inhibin has been postulated as a marker of ovarian tumors (13, 14) and the synthesis of inhibin subunits was demonstrated in these tumors from early to late stages of development, coinciding with SNAP-25 expression, suggesting that these may be related events, although the co-expression of both proteins in the same cells remains to be demonstrated.

In conclusion, various immunocytochemical parameters, described in the normal ovary, were detected in the intrasplenic ovarian tumor. Gonadotropins probably play a fundamental role inducing tumor growth and in maintaining the expression of characteristic proteins, even after 7 months of development, while avoiding apoptosis at the same time. The results obtained indicate that luteomas most likely develop from unruptured follicles by hypertrophy and proliferation of follicular cells and that they represent a highly differentiated non-metastatic tumor model, in which many traits normally observed in the ovary are conserved.

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