Germinal tumor invasion and the role of the testicular stroma

ALEJANDRO DÍEZ-TORRE¹, UNAI SILVÁN¹, OLIVIER DE WEVER², ERIK BRUYNEEL², MARC MAREEL² and JUAN ARÉCHAGA¹

¹Department of Cell Biology and Histology, School of Medicine and Dentistry, University of the Basque Country, Leioa, Vizcaya, Spain and ²Laboratory of Experimental Cancerology, Department of Radiotherapy and Nuclear Medicine, Ghent University Hospital, Ghent, Belgium

ABSTRACT  Testicular germ cell tumors (TGCTs) are the most frequent neoplasia among young people and their incidence has grown very quickly during recent decades in North America and Europe. Many studies have been carried out in order to elucidate the factors involved in the appearance and progression of these tumors. Little is known about the role of cancer cell-stroma crosstalk in TGCT invasive processes. Here, we review several factors which may be implicated in germ cell tumor progression, such as matrix metalloproteinases, insulin-like growth factor, transforming growth factor beta, the cadherin/catenin complex and integrins. Paradoxically, some of these molecules are also involved in the regulation of normal testicular function. Finally, we discuss prospects for future research on the role of the stroma in the progression and differentiation of male germ cell tumors.

KEY WORDS: germinal tumor, embryonal carcinoma, teratocarcinoma, ES, EG, stroma-cell interaction

Introduction

In order to invade surrounding tissues and produce metastasis, cancer cells detach from the primary tumor, break through the basement membrane and reach the circulation. Cell-cell and cell-matrix adhesion molecule alterations, motility factors and degradation of extracellular matrix (ECM) by proteolytic enzymes are needed for these processes. Recent advances in cancer research have highlighted the important role of the cancer-stroma interaction in the regulation of invasive processes. However, very little is known about the role of the stroma in testicular germ cell tumor invasion. The aim of this paper is to review current knowledge about factors involved in tumor invasion in general and the possible role of the testicular stroma in the case of germinal tumors of the testis.

Mouse seminiferous tubules are isolated from the stroma by a monolayer of peritubular myoid cells with basement membrane on both sides. Many cell types are present in the stromal compartment of the testis, including Leydig cells, which are the most abundant and constitute a specific cell population. The main function of Leydig cells is the synthesis of testosterone, which among other things stimulates spermatogenesis. Histopathology of testicular germ cell tumors frequently reveals the proliferation of both myoid cells and Leydig cells, which suggests a possible implication of these cells in tumor progression; new blood vessel formation has also been observed. In the testicular stroma other cell types are present, such as fibroblasts, macrophages, mast cells, lymphocytes and endothelial cells. Each of these may play a role in germ cell tumor invasion, as they do in other kinds of tumors, by secreting proteolytic enzymes, by triggering pro-invasive signals or by facilitating the extravasation of cancer cells to the circulation (Fig. 1).

Testicular germ cell tumors (TGCTs) represent 95 % of testicular neoplasias, but only ~2 % of all cancers in males. Histologically they can be classified as seminomas which are made up of undifferentiated germ cells, and non-seminomas which are made up of undifferentiated multipotent cells (embryonal carcinoma) and derived differentiated populations. Examples of the latter include embryoid bodies and/or other more differentiated tissues com-

Abbreviations used in this paper: dbcAMP, dibutyryl cyclic AMP; EC, embryonal carcinoma; ECM, extracellular matrix; EG, embryonic germ; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; FSH, follicle stimulating hormone; GH, growth hormone; IGF, insulin-like growth factor; IL, interleukin; LH, luteinizing hormone; MMP, matrix metalloproteinase; PDGF, platelet derived growth factor; PGC, primordial germ cell; SF/HGF, scatter factor/hepatocyte growth factor; TGCT, testicular germ cell tumor; TGF-β, transforming growth factor β; TIMP, tissue inhibitors of metalloproteinases.

*Address correspondence to: Dr. Juan Aréchaga. Department of Cell Biology and Histology, School of Medicine and Dentistry, University of the Basque Country, E-48940 Leioa, Vizcaya, Spain. Fax: +34-94-601-3266. e-mail: gcparmaj@lg.ehu.es*
posed of somatic (teratocarcinoma), trophoblastic (choriocarcinoma) or endodermic (yolk sac tumor) cells. However, although their histogenesis is a matter of debate (Damjanov, 1991), both tumors have as a common precursor the carcinoma-in-situ of the testis, which is derived from primordial germ cells (PGCs) at early stages of embryonic development (Skakkebaek et al., 1987; Jorgensen et al., 1995). At birth, there are a low number of carcinoma-in-situ cells in the seminiferous tubules which remain latent till puberty, when they begin spreading along the basement membrane of the germinal epithelium, probably due to the increase in the levels of sexual hormones. The factors which trigger the transition from carcinoma-in-situ to invasive tumor (Fig. 1) can be both genetic and environmental in nature. The genetic alteration most clearly involved in this progression is the overrepresentation of the short arm of chromosome 12 (Roelofs et al., 2000; Looijenga et al., 2003), present in the invasive and adjacent cells, but not in early stages of carcinoma-in-situ. This suggests that genes in this region could be involved in tumor progression. One of these genes is the Defender against Apoptotic Death-Related gene (DAD-R), whose function is not yet known. However, its homologous gene Defender against Apoptotic Death-1 (DAD-1) has been shown to be associated with protection against apoptosis (Zafarana et al., 2002). Among testicular tumors, embryonal carcinoma is one of the most aggressive because of its high metastatic potential. It produces small tumors of heterogeneous appearance and maintains its ability to differentiate into other pathological variants, which include embryonal (in teratomas and teratocarcinomas) and extraembryonal (trophoblast and yolk sac) tissues. Because of the morphological and biochemical similarities between embryonal carcinoma and embryonic stem cells, such as their ability to differentiate into tissues derived from the three germ layers, they have become frequently used as models in stem cell differentiation studies.

In the middle of the 20th century the teratocarcinoma, whose stem cells are embryonal carcinoma cells, became a very important research model in Embryology. Embryonal carcinoma cells were isolated from both spontaneous (Kahan and Ephrussi, 1970) and experimentally induced teratocarcinomas (Evans, 1972; Berstine et al., 1973). The experimental induction of these tumors was achieved by means of transplantation of gonadal ridges or experimentally induced teratocarcinomas (Evans, 1972; Berstine et al., 1973). The experimental induction of these tumors was achieved by means of transplantation of gonadal ridges or whole embryos, up to 12.5 days post coitum, into extra-uterine sites, such as the kidney capsule (Solter et al., 1970) or testis (Stevens, 1970). The germinal origin of teratocarcinomas was demonstrated using these techniques (Stevens, 1970). Nowadays...
many embryonal carcinoma cell lines are available. The F9 cell line is one of them which was isolated from the teratocarcinoma OTT-6050 (Berstine et al., 1973), which in turn was generated by the transplantation of a 6 day embryo into a 129/Sv strain mouse testis, a mouse strain which has a high incidence of spontaneous teratomas and, much less frequently, teratocarcinomas (Stevens and Hummel, 1957; Jiang and Nadeau, 2001). Because F9 is a nullipotent cell line which shares many characteristics with cells of the early embryos, it is an appropriate model for studying the molecular mechanisms of differentiation (Lehtonen et al., 1989; Alonso et al., 1991). P19 is another embryonal carcinoma cell line, isolated from an experimental teratocarcinoma induced in the C3H/He mouse strain (McBurney and Rogers, 1982; McBurney, 1993) and characterized by its capacity to differentiate into the three germ layer-derived tissues. Human embryonal carcinoma cell lines have also been isolated, for example Ntera-2 (Andrews et al., 1984).

Whereas seminomas are functionally equivalent to PGCs, non-seminomas, such as embryonal carcinoma, are more similar to embryonic stem cells (Oosterhuis and Looijenga, 2003). Embryonic stem cell lines can be obtained from the inner cell mass of preimplanted blastocysts and cultured on a feeder layer of fibroblasts (Evans and Kaufman, 1981). In this way, cell colonies can be maintained undifferentiated in culture for a long time. The AB1 cell line was produced by this technique. On the other hand, embryonic germ cell lines have been obtained directly from PGCs cultured in vitro. The EG-1 cell line, for example, was obtained from PGCs of 129/Sv mouse embryos (Steward et al., 1994).

Matrix metalloproteinases

Proteinases play an essential role in tumor invasion, taking part in several steps of the metastatic process, such as angiogenesis, local invasion or the intra- and extravasation, into or from the circulation. The proteinases involved in these processes include cathepsins (Thorpe et al., 1989), plasminogen activators (Duffy et al., 1988) and matrix metalloproteinases (MMPs; Folgueras et al., 2004). Recently, the role of MMPs as mediators of the cancer-host interaction has been reviewed (Lynch and Matrisian, 2002). The MMPs constitute a family of zinc-dependent endopeptidases, the majority of which appear in the form of latent pro-enzymes which need the proteolytic degradation of a pro-domain to become active. The MMPs can be secreted, although they need to be tethered close to the membrane by specific receptors to have focused proteolytic activity, or appear associated to the membrane by means of a transmembrane domain. Depending on their substrate preference they are classified into collagenases, gelatinases, stromelysins and membrane type MMPs (MT-MMPs).

Cancer cells were considered to be the main producers of proteinases, responsible for the degradation of the ECM during invasion. In fact, the first immunohistochemistry experiments on breast carcinoma showed that MMP-2 was present in cancer cells at the invasion front (Barsky et al., 1993). Protease expression by cancer cells has also been observed in vitro invasion assays with collagen type I and Matrigel (reconstituted basement membrane) as substrates. For example, we recently observed that the invasive capacity of the murine embryonal carcinoma P19 cells in Matrigel decreased by 80% when treated with a protease inhibitory mixture consisting of galardin, aprotinin and leupeptin (data not shown).

Using gelatin zymography, we observed pro-MMP-2 expression in the cell lines P19 and F9, in the human embryonal carcinoma cell line Ntera-2, in the mouse embryonic stem cell line AB1 and in the embryonic germ cell line EG-1. In the case of P19 and AB-1, the expression of pro-MMP-9 was also detected, but at a much lower level than that of pro-MMP-2. In contrast, the Ntera-2 cell line was found to express both the pro-MMP-2 and pro-MMP-9 proteinases at similar levels and it is the only one which produces the active form of MMP-2 (Fig. 2).

The main role of cancer cells in the secretion of proteinases was reconsidered when it was shown by hybridization techniques that stroma cells are the main source of MMPs. Several papers showed that MMP transcripts appear mostly in stromal cells (Okada et al., 1995; Heppner et al., 1996; Uria et al., 1997). MMP-11, for instance, has been localized specifically in breast tumor associated fibroblasts, but not in normal breast fibroblasts (Basset et al., 1990). MMP-7 is an exception as it is a unique MMP which seems to be expressed preferentially by cancer cells in most cases. We did not detect expression of MMP-7 (matrilysin) in F9 or P19 embryonal carcinoma cell lines using casein zymography (data not shown). Distinct works have demonstrated that MMP-7 expression by cancer cells requires them to interact with surrounding stromal cells (Bair et al., 2001).

Therefore the proteolytic process is achieved jointly by cancer cells and stromal cells. One example is proMMP-2 derived from fibroblasts, which is activated by the MT1-MMP/TIMP-2 complex present in the membrane of carcinoma cells (Strongin et al., 1995; Sato et al., 1999; Mitra et al., 2003), hence facilitating cancer invasion, although others mechanisms have been proposed for MMP-2 activity on the cancer cell surface (Emmert-Buck et al., 1995; Brooks et al., 1996). It has been observed recently that the levels of proMMP-2, MT1-MMP and TIMP-2 in rat Sertoli cells in culture are increased by treatment with follicle-stimulating hormone (FSH) (Slongo et al., 2002). It is also known that the expression of proMMP-2 in peritubular myoid cells is about 25-fold higher than that of Sertoli cells in culture. This suggests that myoid cells provide pro-invasive information by the secretion of proteinases. This secretion is upregulated by concanavalin A, dibutyryl cyclic AMP (dbcAMP) and IL-1 (Hoeben et al., 1996).

![Fig. 2. Zymography. Culture media harvested from AB1 murine embryonic stem cells, EG-1 murine embryonic germ cells, F9 and P19 mouse embryonal carcinoma cells and human embryonal carcinoma cells Ntera-2 were analyzed by gelatin zymography. All the cell lines expressed pro-MMP-2, although F9 and EG-1 conditioned media had to be concentrated five fold more to detect proteolytic activity. Pro-MMP-9 activity was identified in AB1 and F9 cell lines but with a weak signal, while Ntera-2 produces both pro-MMP-2 and pro-MMP-9 at similarly high levels. Interestingly, the Ntera-2 cell line is the only one which expressed the active form of MMP-2 (Lower molecular weigh band).](image-url)
In non-pathological situations, proteolytic activity is strongly regulated to avoid tissue damage. This regulation may appear at the transcriptional or at the translational levels of MMP processing by growth factors or cell-to-cell or cell-to-matrix interactions. Inhibition of the activity of these enzymes can also occur by natural inhibitors called tissue inhibitor of metalloproteinases (TIMP). TIMPs are expressed in a wide variety of cell types and are found in the majority of tissues and corporal fluids. There are four known TIMPs, designated TIMP-1, -2, -3 and -4. TIMPs form non-covalent stoichiometric 1:1 complexes with MMPs or pro-MMPs and inhibit or regulate the enzymatic activity process in this way (Gomez et al., 1997). TIMP-1, -2 and -4 are found in a soluble form, while TIMP-3 appears closely attached to the ECM. The pattern of TIMP-4 mRNA expression in brain, heart, ovary and skeletal muscle suggests that this inhibitor is an important regulator of ECM remodeling (Leco et al., 1997). TIMP-2 facilitates the activation of MMP-2, joining pro-MMP-2 (Howard and Banda, 1991) and TIMP-1 preferentially forms complexes with pro-MMPs and pro-MMPs and inhibit or regulate the enzymatic activity process in this way (Gomez et al., 1997). TIMP-1, -2 and -4 are found in a soluble form, while TIMP-3 appears closely attached to the ECM. The pattern of TIMP-4 mRNA expression in brain, heart, ovary and skeletal muscle suggests that this inhibitor is an important regulator of ECM remodeling (Leco et al., 1997). TIMP-2 facilitates the activation of MMP-2, joining pro-MMP-2 (Howard and Banda, 1991) and TIMP-1 preferentially forms complexes with pro-MMP-9 (Wilhelm et al., 1991). TIMP-2 and -3, but not TIMP-1, are effective inhibitors of MT-MMPs (Bode and Maskos, 2001). The expression of TIMP-2 is constitutive, while TIMP-1 expression is induced by growth factors such as bFGF, PDGF and EGF or cytokines, such as IL-1 and IL-6. The expression of TIMPs and of MMPs is increased by some of these factors, but in the case of TGF-β, retinoids and erythropoietin, TIMP synthesis decreases and TIMP-1 synthesis increases (Clark et al., 1987; Overall, 1994).

TIMPs carry out several functions. Many of them are associated with their inhibition of MMPs, but they also present functions which are independent of MMP. Regulation of cellular growth (Bertaux et al., 1991), prevention of apoptosis (Lee et al., 2003b) and inhibition of angiogenesis (Seo et al., 2003) stand among these functions. The promoting activity of cellular growth is common, at least to TIMP-1, -2 and -3 and several results point to the fact that they exert this function independently of the inhibition of MMPs (Bertaux et al., 1991; Kikuchi et al., 1997). Other authors have described TIMP-1, -2 and -4 as growth inhibitors in some cell types, among which several carcinomas are found (Guedez et al., 2001; Celiker et al., 2001). Proteolytic activity has been found to be required for the angiogenesis process. New blood vessels formation is necessary for tumor growth and metastasis and it has recently been shown that the ets-1 transcription factor is not only involved in the induction of MMP expression, but it also enhances angiogenesis in metastatic testicular germ cell tumors (Adam et al., 2003).

**Growth factors**

Several growth and motility factors have been studied because of their potential relation with tumor development (Mueller et al., 2003; Opdenakker and Van Damme, 2004). These factors can regulate distinct processes involved in invasion, such as cell growth and differentiation, proteolytic activity, cell-cell and cell-matrix adhesion, cell motility and stromal reaction. Insulin-like growth factors (IGFs) and transforming growth factors β (TGF-βs) are known to participate in the regulation of testicular function and both systems have been related with tumor progression. IGF-I and II are peptide hormones of ~7 kDa. Both are synthesized and secreted by many tissues and produce a wide range of responses mainly related to growth and differentiation in several cell lines. They can be transported by blood to distant sites, acting as endocrine hormones. In other cases, they can have local effects acting in a paracrine or autocrine way. Two kinds of receptors for IGFs and six different IGF binding proteins (IGFBP), responsible for the regulation of the IGF system, have been identified (Lowe, 1996; Clemmons, 1998). Both IGF-I and IGF-II are synthesized locally in the testis by Sertoli cells, Leydig cells, germ cells and peritubular cells and the expression of IGF-I is stimulated in vitro by treatments with FSH, LH and GH (Vannelli et al., 1988; Dombrowicz et al., 1992). Both IGF-I and IGF-II participate in the local regulation of testicular function. In the interstitium, IGF-I stimulates testoste- one synthesis by Leydig cells (Lin et al., 1986) and in the seminiferous tubules, it has an effect on Sertoli cells, increasing lactate synthesis (Oonk and Grootegoed, 1988) and glucose transport (Oonk et al., 1989), both processes needed for germ cell维持ance. In spermatogonia, IGF-I stimulates DNA synthesis (Söder et al., 1992). It is known that IGF-II participates in embryonic and fetal development and it can enhance the proliferation of spermatogonia in vitro, but its postnatal function is still not clear.

Many observations have shown that IGF-I and II are involved in cancer pathobiology. They are associated with a higher risk of tumor development and with more invasive behavior of cancer (Rosen and Pollak, 1999). It is known that many oncogenes enhance the expression of IGF system components. In contrast, this system can be inhibited by tumor suppressor genes (Baserga et al., 1997). Other evidence demonstrates the association between the IGF system and cancer progression (Grimberg, 2003). Several studies identify the IGF system as promoter of the expression of many extracellular matrix proteinases, mainly of the MMP group. The IGF-I receptor (IGF-IR) has been recognized as a regulator of MMP-2; this protease is commonly implicated in the degradation of the basal membrane and its up-regulation has been associated with angiogenesis, tumor invasion and metastasis.

Occasionally IGF-I signaling can be conflicting. For example, when the H-59 lung carcinoma cell line is treated with IGF-I at 10 ng/ml, the synthesis and activation of MMP-2 is enhanced via the PI3-K/Akt signal pathway, but at a higher IGF-I concentration (100 ng/ml), it can inhibit the synthesis of MMP-2 in this cell line via the Raf/ERK pathway. Thus, the final effect of the IGF-IR on MMP-2 activity may depend on other factors, such as ligand availability, which shift the balance toward the activation or the inhibition pathway (Zhang et al., 2004). We observed in Matrigel invasion experiments that treatments with wortmannin, a PI3-K inhibitor, reduces the number of invasive cells of the P19 embryonal carcinoma cell line by up to 70 % (data not shown), but the mechanism of this inhibition remains unclear. The IGF-IR regulates MT1-MMP expression and so the MMP-2 activation process (Zhang et al., 2003). High levels of circulating IGFs and low levels of IGF Binding Protein 3 (IGFBP-3) have recently been shown to be associated with a higher risk of developing colon, breast, prostate and lung cancer, which are known to produce MMP-7. IGFBP-3 regulates the IGF system, inhibiting the binding of IGF-I to its receptor. The degradation of IGFBP-3 by MMP-7 increases the levels of IGF-I associated with the IGF-IR and consequently the Akt signal pathway is activated, thereby preventing apoptosis and promoting cell survival (Miyamoto et al., 2004). High levels of IGF-IR expression have been found to be related to a more invasive behavior in tumors such as human and canine osteosarcomas (MacEwen et al., 2004) and thyroid carcinomas (Gydeee et al., 2004). IGF-IIIR is supposed to be a tumor suppressor as it is usually mutated in
malignant human tumors. It has been demonstrated that downregulation of IGF-IIIR enhances growth in cancer cells by maintaining IGF-II, which activates growth promoting signals through IGF-IR (Osipo et al., 2001). In human lung tumors it has been observed that IGF-I, IGF-II and other growth factors are implicated in the upregulation of MMP-2 and MMP-9 activity and in increased cell migration (Bredin et al., 2003). With regard to the mechanism of IGF-I activity, a recent study has shown that α-catenin is necessary for IGF-I-induced cell migration in the HCT-8 human colon cancer cell line, while E-cadherin, but not α-catenin, is required for the induction of invasion (Andre et al., 2004).

The role of IGF-I in testicular germ cell tumors has been studied. It has been found to be expressed by carcinoma-in-situ cells which also express high levels of IGFBP-5. The latter may regulate the activity of IGF-I and provide a proliferative advantage to the tumor cells (Drescher et al., 1997).

TGF-βs are peptide hormones which are also involved in the regulation of tumor development and progression (Akhurst and Derynck, 2001). These factors are homodimers of about 25 kDa, consisting of two identical subunits of 12.5 kDa each. These factors acts by means of two types of TGF-β receptors (TβR) with Ser/Thr kinase activity. Both TβR-I and TβR-II are single-chained integral membrane glycoproteins which form homodimers when they are joined to TGF-β; the resulting multiprotein complex induces a specific downstream signaling pathway (Massagué, 2000). TGF-β has other receptors known as TβR-III and TβR-IV (endoglin), but these receptors do not have kinase activity and act just as accessory proteins which facilitate TGF-β signaling (López-Casillas et al., 1993; Sankar et al., 1995; Barbara et al., 1999). TGF-α and TGF-β were isolated as two factors which could induce a transformed phenotype in normal rat fibroblasts (de Larco and Todaro, 1978). While TGF-α is a mitogenic factor, TGF-β acts as a potent growth inhibitor for most cell types (Coffey et al., 1988). Its inhibitory effect on cell proliferation together with its ability to induce apoptosis made TGF-β a potential tumor suppressor. This role of TGF-β is supported by many observations (Akhurst et al., 1988; Cui et al., 1995); thus, the downstream perturbation of TGF-β signaling leads to tumor outgrowth and malignant progression. Mutations in TGFBR2 (which encodes the type II TGF-β receptor) are present in tumors from patients with hereditary non-polyposis colorectal cancer (HNPPC) (Markowitz et al., 1995) and other mutations such as those found in TGFBR1 (which encodes the TGF-β type I receptor), MADM2 and MADM4 (encoding Smad2 and Smad4 proteins respectively), have been found in several solid tumors such as pancreas and breast carcinomas (Massagué et al., 2000). Nevertheless, most human tumors do not have any alteration in the TGF-β system and retain a correctly functional signaling pathway. However they are still resistant to its growth inhibitory effect, while they conserve and even increase other effects (Calonge and Messagué, 1999; Lehmann et al., 2000).

Moreover, tumors with loss-of-function mutations in the TGF-β system have a better prognosis than those which preserve a functional signaling pathway. Members of this last group, despite presenting a lower proliferation rate, show much more invasive behavior and higher metastatic potential (Tsushima et al., 1996; Wikstrom et al., 1998). This was demonstrated by an experiment in which a colon cancer cell line lacking TGF-β receptor II expression was transfected with functional TGF-βRII cDNA. The result was a reduction in the growth rate and a strong stimulation of the

Fig. 3. Fast aggregation assay. Representation of particle diameter (µm) vs. relative volume (%) of aggregates at time = 0 (blue), time = 30 min without treatment (red) and time = 30 min incubating with E-cadherin blocking antibodies (green). The graphs correspond to the AB1 murine embryonic stem cell line (A), the EG1 embryonic germ cell line (B), the murine embryonal carcinoma cell lines F9 (C) and P19 (D) and the human embryonal carcinoma Ntera-2 (E). The human cell line did not aggregate, while murine embryonal carcinomas aggregated, even in the presence of E-cadherin blocking antibodies, suggesting that this cadherin is not the main one responsible for cell-cell adhesion. In contrast, the aggregation capacity of AB1 and EG-1 were sensitive to E-cadherin blocking treatment.
invasive and metastatic capacity (Oft et al., 1998). It has also been observed that mammary adenocarcinoma cells treated with TGF-β undergo an increase in their metastasis formation ability when they are injected in syngenic rats (Welch et al., 1990). These results imply that TGF-β can act as both a tumor suppressor at early stages of tumorogenesis and as invasion stimulator, during advanced stages. The mechanisms employed by TGF-β to stimulate tumor invasion include the induction of epithelial to mesenchymal transformation, enhancement of proteinase synthesis and activity, angiogenesis induction and immunosuppressive activities (Akhurst and Derynck, 2001).

The three isoforms of TGF-β (β1, β2 and β3) have been found to be expressed in the testis. The producer cells and the predominant isoform expressed depend on the stage of the tubule and on the age of the animal (Mullaney and Skinner, 1993; Teerds and Dorrington, 1993). Sertoli cells and peritubular myoid cells express TGF-β1 throughout testicular development, while in Leydig cells, it is only detected at fetal and neonatal stages. During these stages, TGF-β2 is the main isoform in the testis, located in Sertoli and Leydig cells (Olaso et al., 1997; Teers and Dorrington, 1993). As for the receptors of TGF-β, both TβR-I and -II are expressed by Sertoli and Leydig cells (Le Maguereesse-Battistoni et al., 1995). TβR-II is also located in spermatogonia (Mullaney and Skinner, 1993). Concerning TGF-β functions in the testis, it has been demonstrated that TGF-β enhances testosterone production by fetal Leydig cells at low concentrations, although it inhibits this production when present at higher concentrations (Benahmed et al., 1989). TGF-β plays a fundamental role in the determination of germ cell number in the seminiferous epithelium, as it reduces their proliferation and induces apoptosis (Prepin and Le Vigouroux, 1997; Olasco et al., 1998). The ECM of the semiferous tubules is regulated by the interaction of proteinases, proteinase inhibitors and several cytokines, including TGF-β and tumor necrosis factor alpha (TNF-α). In Sertoli cells, TGF-β stimulates the synthesis of proteoglycan (Panthou et al., 1994). Moreover, it reduces proteolytic activity in the ECM, increasing the production of Plasminogen Activator Inhibitor (PAI-1) (Le Maguereesse-Battistoni et al., 1998). TGF-β also regulates the action of FSH on Sertoli cells (Morera et al., 1992). It has been observed that TGF-β has chemoattractant effects on PGCs. For this reason, it is considered to be one of the cytokines implicated in their migration towards the gonadal ridges (De Felici and Pesce, 1994). TGF-β3 also plays an important role in the migration of the preleptotene and leptotene spermatocytes through the blood-testis barrier, because it regulates its opening and closing (Lui et al., 2003).

TGF-β1 is known to mediate fibroblast to myofibroblast differentiation (Evans et al., 2003) and acts as a fibroblast chemoattractant at the invasion front (De Wever and Mareel, 2003). Myofibroblasts were described as smooth muscle-like fibroblasts (Gabbiani et al., 1971) and their paracrine function has been widely reviewed (Powell et al., 1999). The amount of myofibroblasts found in malignant tumors is much higher than in non-invasive ones and they are localized predominantly at the invasion front (Nakayama et al., 1998). This finding suggests that cytokines expressed by tumor cells, may induce fibroblast to myofibroblast differentiation. The role of myofibroblasts in the invasion process has recently been reviewed (De Wever and Mareel, 2002). It has been shown that factors produced by myofibroblasts, such as tenascin-C and scatter factor/hepatocyte growth factor (SF/HGF) provide proinvasive signals via the Rho family of GTPases in human colon cancer cells (De Wever et al., 2004). The role of epithelial-mesenchymal interaction in the induction of tenascin expression has been reviewed (Ekblom and Auferheide, 1989).

### Adhesion molecules and cytoskeleton

As mentioned previously, cancer cells have to separate from the primary tumor and migrate through the ECM to reach the circulation before giving rise to metastatic tumors. In this process both cell-cell and cell-matrix adhesion molecules play a key role. The four groups of molecules involved in cell-cell and cell-matrix adhesion are cadherins, integrins, members of the immunoglobulin superfamily and selectins (Pignatelli and Vessey, 1994; Beavon, 2000).

Concerning cell-cell adhesion, the E-cadherin/catenin complex is of particular interest. E-cadherin is a transmembrane glycoprotein of 120 kDa belonging to the cadherin type I superfamily (Nollet et al., 2000), which is made up of calcium-dependent cell-cell adhesion molecules. The adhesion mediated by these molecules is generally homophilic (cadherin-cadherin) and homotypic (between the same cell type), even though there are exceptions, such as the adhesion of epidermal Langerhans cells to keratinocytes or between epithelial cells and T lymphocytes (Tang et al., 1993; Cepek et al., 1994). E-cadherin is made up of an extracellular N-terminal region comprising five subdomains with cadherin-motives tandemly repeated, one transmembrane region and a highly conserved cytoplasmic C-terminal region. The cytoplasmic domain is linked to the actin cytoskeleton via the catenins α, β and γ (Tucker and Pignatelli, 2000; Nagafuchi and Takeichi, 1988). This interaction suggests that E-cadherin has not only a structural function, but it is also implicated in signaling pathways (Chausovsky et al., 2000). Because of its role in the maintenance of normal epithelial architecture, E-cadherin has been identified as a potential tumor and invasion suppressor (Frixen et al., 1991). Furthermore, it has been demonstrated that E-cadherin acts as a growth suppressor via up-regulation of cyclin-dependent kinase p27 (St. Croix et al., 2000). A correlation between cancer aggressiveness and alterations in the E-cadherin/catenin complex has been reported (Bracke et al., 1996; Debruyne et al., 1999). The causal relationship between E-cadherin and in vivo invasion was demonstrated using a transgenic mouse model presenting a high incidence of pancreatic β-cell adenoma. The loss of E-cadherin mediated cell-cell adhesion induced the adenoma to invasive carcinoma transition, while overexpression of E-cadherin diminished the appearance of the invasive phenotype (Perl et al., 1998).

Using the Fast Aggregation Assay (Bracke et al., 1994) in the presence and absence of specific anti-E-cadherin antibodies, we observed that the embryonic germ cell line EG-1 and the embryonic stem cell line AB1 present aggregation which is sensitive to E-cadherin blocking, while the two murine embryonal carcinoma cell lines F9 and P19 still aggregate even in the presence of these antibodies. This result suggests that E-cadherin is not the principle factor which is responsible for intercellular adhesion in these cell lines. Rather, another cell-cell adhesion molecule is necessarily involved and is sufficient to maintain aggregate formation ability by itself. We did not obtain aggregates in experiments involving the...
Germinal tumor invasion

human embryonal carcinoma Ntera-2, suggesting that E-cadherin might not be functional in this cell line (Fig. 3). Recently, high levels of a soluble fragment of E-cadherin, resulting from cleavage by MMPs (Noë et al., 2001) produced by cancer or host stromal cells, have been detected in serum from cancer patients and in the conditioned medium of some cancer cell lines (Wheelock et al., 1987). The 80 kDa E-cadherin fragment has been identified as MMP expression promoter in human lung cancer cells (Nawrocki-Raby et al., 2003).

The switch from E-cadherin to N-cadherin (Cavallaro et al., 2002) in cancer cells has also been described. This switch facilitates the interaction of these cells with the host stromal cells and hence the invasion process (De Wever and Mareel, 2003). β-catenin is an element of both the E-cadherin/catenin complex (Ozawa et al., 1989) and the Wnt signaling pathway, in which β-catenin may have a tumorigenic effect (Behrens et al., 1996; Willert and Nusse, 1998). Mutations in β-catenin phosphorylation sites (Iwao et al., 1998) or in β-catenin binding sites of the adenomatous polyposis coli (APC) complex (Munemitsu et al., 1995) lead to a nuclear accumulation of β-catenin in association with lymphocyte enhancer factor/T-cell factor (LEF/TCF). This leads to the formation of a transactivator complex which is involved in the expression of several genes.

**Fig. 4.** Actin cytoskeleton stained with Falloidin-FITC in the murine cell lines AB1 (embryonic stem cells), EG-1 (embryonic germ cells), F9 and P19 (both embryonal carcinoma cells), seeded over different substrates. AB1, EG-1 and F9 showed weak adhesiveness to collagen type I presenting a round shape. AB1 and F9 produced fillopodia and lamellipodia on both fibronectin and laminin, while EG-1 presented these only on laminin, showing the same behavior on fibronectin as on collagen type I. P19 had the same adhesiveness on the three substrates and these cells produced extensions in all of them.
implicated in cancer development, such as MMP-7 (Crawford et al., 1999), myc (He et al., 1998), cyclin D1 (Lin et al., 2000) and components of the AP-1 transcription complex (Mann et al., 1999).

It has been recently demonstrated that in the testis, both Sertoli and germ cells express E-cadherin, N-cadherin, α-catenin, β-catenin and p120ctn. The co-localization and the formation of a complex between N-cadherin and β-catenin at Sertoli-Sertoli and Sertoli-germ cell adherens junctions in the lower third of the seminiferous epithelium has also been shown. These assays demonstrate that the cadherin/catenin complex is involved in the assembly of functional adherens junctions between Sertoli and germ cells. (Lee et al., 2003a). Despite these results, a study of E-cadherin expression in testis tumors carried out a few years earlier failed to detect E-cadherin on normal germ cells, but it was detected on 18.8 % of studied seminomas and 62.5 % of non-seminomas (Saito et al., 2000). In the same study α-catenin was not detected in any of the studied seminomas and in only 25 % of non-seminomas, while β-catenin was detected in 71.4 % of seminomas and 81.2 % of non-seminomas. E-cadherin has also been identified as an important regulator of PGC migration and homing at gonadal ridges and may modulate PGC development (Di Carlo and De Felici, 2000). N-cadherin has been located on the surface of spermatogonia, primary spermatocytes and endothelial cells, but not on peritubular and Leydig cells (Anderson et al., 1994). P-cadherin is detected in Sertoli cells up to postnatal day 8 but not later, while this cadherin starts to be expressed in peritubular cells at the same time as β-catenin on days 12 and 15. The formation of the P-cadherin/β-catenin complex in peritubular cell contact areas coincides with the formation of a mature pattern of actin filament organization in these cells (Lin and DePhilip, 1996).

Cell-matrix adhesion proteins are also involved in tumor invasion. The role of integrins in this process has been recently reviewed (Hood and Cheresh, 2002). The ECM has to be degraded to allow sufficient but not excessive cell passage, otherwise cellular traction would be lost (Sheetz et al., 1999). Integrins are not only involved in this traction movement but also take part in signaling pathways which regulate the migration and invasion processes. Integrins are a family of glycoproteins which form heterodimeric receptors for ECM molecules. There are 18 α-subunits and 8 β-subunits which can form at least 25 different heterodimers; each of them recognizes a specific set of ligands (van der Flier and Sonnenberg, 2001). Integrins are associated with the actin cytoskeleton via several proteins present in focal contacts (Sastry and Sonnenberg, 2001). Integrins also activate kinases which regulate stress-fiber formation, cell shape and migration. In cancer cells, important alterations in the level of expression of integrins and in their affinity for extracellular matrix ligands have been reported (Mizejewski, 1999).

A study of teratoma induction by injection of normal ES cells and β1 integrin-lacking ES cells demonstrated that β1 integrin is necessary for teratoma growth and angiogenesis (Bloch et al., 1997). It has also been demonstrated that β1 integrin as well as E- and N-cadherins are involved in MMP-7 induction in cocultures of oral squamous cell carcinoma cell line SCC-25 with human foreskin fibroblasts (Bair et al., 2001). Increased expression of β1 and α5 integrins have been found to be associated with lymph node metastasis in non-small lung cancer (Han et al., 2003). We observed that the PGC-derived EG-1 cell line adheres preferentially to laminin and forms lamellipodia on this substrate, while these cells have a round shape and adhere deficiently to fibronectin and collagen type I. This result is consistent with previous observations regarding the affinity of PGCs and spermatogonial stem cells for laminin (García-Castro et al., 1997; Shinohara et al., 1999). In the same experiment, we saw that cells of the F9 embryonal carcinoma cell line have the same behavior as AB1 embryonic stem cells, with round shape and low adhesiveness on collagen type I and formation of prolongations on fibronectin and laminin. P19 cells adhere in a similar way to the three substrates forming prolongations in all of them (Fig. 4). The α6β1 integrin is known to act as a laminin receptor (Hynes, 1992) and it has also been identified as a mouse spermatogonial stem cell surface marker (Shinohara et al., 1999).

In one of the first papers about the tumor-extracellular matrix interactions in TGCTs it was shown that malignant intratubular germ cells express both α6 and β1 integrin subunits and also α3, the loss of which is associated with progression of intratubular neoplasia to invasive seminoma (Timmer et al., 1994). In the rat testis, the β1 integrin has been located in peritubular cells, basement membrane, lamina propria and the basal cytoplasm of Sertoli...
cells. The α1 subunit was also detected in peritubular cells and lamina propria. The α9 subunit was only detected in the basement membrane and in peritubular cells (Giebel et al., 1997). The β1 subunit has been shown to participate in the regulation of the adherens junctions between Sertoli cells and germ cells (Siu et al., 2003). These junctions have to be disrupted in germ cell malignancies to allow the invasion process. Indeed the β1 subunit seems to be downregulated in diffuse seminomas compared with non invasive tumor (Restucci et al., 2000).

Integrins and other cell-surface receptors are involved in the activation of Rho small GTPases (Ren et al., 1999; Ishiguro et al., 2000). The Rho family of GTPases, including Rho, Cdc42 and Rac, can regulate changes in the cytoskeletal organization needed for the initiation of migration (Nobes and Hall, 1995). The role of Rho family GTPases in invasion has already been reviewed (Sander and Collard, 1999). In vivo experiments have demonstrated that Rho proteins are involved in the acquisition of the metastatic phenotype (Del Peso et al., 1997). The induction of Rho activity by lysosphosphatidic acid in a hepatoma cell line stimulated migration and invasion through Matrigel in vitro (Wang et al., 2004). Recently, the Rho signaling pathway has also been found to be related to the progression of testicular germ cell tumors, since in these tumors the expression of RhoA and Rho-kinase mRNA is higher than in the normal testicular tissue (Kamai et al., 2002).

Conclusions and perspectives

The aim of this work is to give an overview of factors potentially implicated in testicular germ cell tumor invasion in order to facilitate future research in this area. Accordingly, we summarize recent advances in cancer invasion research, mentioning specially the implication of tumor-stroma interactions in invasiveness and the role of some factors involved in the invasion process.

Embryonal carcinoma is a type of germ cell tumor which affects mainly adolescents and young adults. They are rare but their incidence has grown fast in recent decades in North America and Europe (Moller and Evans, 2003). Consequently it is of particular interest to understand the mechanisms involved in their invasive behavior. The embryonal carcinoma invasion process is still unknown. Myofibroblast differentiation. However, the role of testicular stroma in the embryonal carcinoma invasion process is still unknown.

Acknowledgements

A. Díez-Torre and U. Silván are Ph.D. students supported by fellowships from the Spanish Ministry for Education and Science and from the University of the Basque Country respectively. This work was supported by a University of the Basque Country Research Grant (#9/UPV0077.327/15304/2003) and by FORTIS VERZEKERINGEN, Brussels, Belgium. We are very grateful to Allan Bradley and the Baylor College for providing the AB1 cell line and to Colin Steward for the EG-1 cell line.

References


